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Deseret Test Center
HQ Pacific Island
Fort Detrick, Md. 21715

ANNUAL
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FY 1969

RCS-MEDDH-288(RI)

UNITED STATES ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

FORT DETRICK, MARYLAND

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U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

FORT DETRICK, FREDERICK, MARYLAND 21701

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FISCAL YEAR 1969

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Project 1B662706A096

1 July 1969

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13. ABSTRACT A report of progress on the research program of the U. S. Army Medical Research Institute of Infectious Diseases on Medical Defense Aspects of Biological Warfare (U) for Fiscal Year 1969 is presented. KEYWORDS: Biological warfare Vulnerability Therapy Prophylaxis Identification Bacterial diseases Rickettsial diseases Host Parasite Biochemistry Pathology Defense Metabolism Virus diseases			

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FOREWORD

This FY 1969 Annual Progress Report is a general review of research activities of the U. S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland, conducted under Project 1B662706A096, Medical Defense Aspects of Biological Agents (U). The project is divided into three tasks:

- 1B662706A096 01 - Vulnerability of Man to Biological Agents.
- 1B662706A096 02 - Prevention and Treatment of Biological Warfare Casualties.
- 1B662706A096 03 - Laboratory Identification of Biological Agents.

Sixteen contracts are currently in effect with educational institutions or industrial firms. Reports are available through DDC.

Tasks are subdivided into work units, each identified by a three digit suffix. Numbers have been assigned in accordance with the following scheme:

General	001-099
Bacterial Diseases	100-299
Rickettsial Diseases	300-399
Viral Diseases	400-699
Mycotic Diseases	700-799
Intoxications	800-899
Contracts	900-999

Three appendices are included covering the Guest Lecture Series, Professional Staff Meetings, Formal Presentations and Briefings and a list of publications of the Institute for the Fiscal Year. An index by authors has been added.

In conducting research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

1 July 1968

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 001: Metabolic Studies in Experimental Disease

Reporting Installation: U. S. Army Medical Research Institute of
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Fort Detrick, Maryland

Divisions: Physical Sciences and Medical

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				NAME: Bostian, K. DA			
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23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Study early changes in metabolism induced by experimental infection.							
24. (U) A variety of techniques are employed to study metabolic changes associated with infection.							
25. (U) 68 07 - 69 06 - Significant decreases in serum Fe and total Fe binding capacity and in serum Zn occur in humans infected with <u>Pasteurella tularensis</u> and attenuated VEE vaccine. Recent animal studies have shown increasing evidence of endogenous pyrogen being involved in these reductions.							
<p>Release of an endogenous pyrogen appears to be involved in the production of these changes. A study to assess the sequential changes in blood pH, PCO-2, PO-2, lactate, pyruvate, and phosphorus during induced infection is in progress.</p> <p>Publication: Fed. Proc. 28:691, 1969 (abstract).</p>							

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 001: Metabolic Studies in Experimental Disease

Description:

To study early changes in metabolism induced by experimental disease.

Progress, Part I:

Preliminary studies on mice infected with the attenuated vaccine strain of Pasteurella tularensis have demonstrated that significant decreases in serum Fe concentration occur early in the course of infection and before onset of detectable illness.^{1/}

The effect of P. tularensis infection on serum Fe and total Fe-binding capacity levels in the human host was studied. Twenty healthy, 20-26 year old male soldiers volunteered to participate in the study. The volunteers comprised 2 groups of 10 men each. Each group consisted of 8 previously vaccinated and 2 nonvaccinated men. All subjects were exposed to aerosols of viable SCHU-S4 strain P. tularensis. Each subject in Group I received approximately 2500 viable organisms, and subjects of Group II approximately 25,000 viable organisms. Blood samples were obtained at 0800 and 2000 hours daily throughout the study and included at least 3 days of preexposure determinations for baseline values. Aliquots of serum were divided for colorimetric and atomic absorption determinations. Based on fever (rectal temperature >100 F) the subjects were divided into 3 clinical response categories: typical illness, mild illness, and asymptomatic.

From this investigation it was shown and confirmed by 2 analytical methods that serum Fe concentrations and total Fe-binding capacity fall early after exposure of volunteers to virulent P. tularensis. Serum Fe fell significantly and consistently below values encompassing normal changes or differences among individuals. The prospective nature of this study and the rapid development of hypoferremia, even in vaccinated subjects who remained asymptomatic, made it evident that serum Fe fell in two distinct phases. The first, or Exposure Phase Hypoferremia, represented a consistent fall of modest magnitude early in the incubation period which seemed to be an early host response to invading microorganisms, a response independent of subsequent illness or its absence. The second, or Illness (Febrile) Phase Hypoferremia, presented as an exaggerated, superimposed response related in timing and magnitude to

the onset and severity of illness. A diurnal variation in serum Fe levels was also found and confirmed in the preexposure period. However, in the postexposure period diurnal variation was found to be reduced or was absent. The possibility was raised that an endogenous mediating factor was released from neutrophilic leukocytes and served to trigger the observed changes in Fe metabolism.

More recently, alterations in serum Fe, Cu, and Zn concentrations were studied prospectively in a group of volunteers receiving Venezuelan equine encephalomyelitis Vaccine, Live, Attenuated, Lot 6. Again, subjects were divided into 3 clinical response groups based on fever.

Significant decreases in serum Fe and Zn concentrations occurred prior to the onset of illness reaching maximum reductions closely corresponding to the febrile response. As in the tularemia study, asymptomatic individuals also showed early moderate reductions. A corresponding rise in serum Cu concentrations were also observed.

Studies on rats, and rabbits have shown increasing evidence that the release of an endogenous mediator from polymorphonuclear (PMN) leukocytes and mononuclear macrophages may be directly or indirectly involved in the production of hypoferremia and serum Zn reductions in the infected host. When adult male rats were given 0.01, 0.1, 1.0, 10, or 100 μ g of Escherichia coli lipopolysaccharide B. intraperitoneally (IP) serum Zn and Fe values fell significantly within 3 hr and became maximally depressed within 9 hr. Reductions in these 2 trace metals showed a linear correlation with the logarithm of the dose. The Zn and Fe depressing effect was mediated by a heat labile endogenous factor. Within 2 hr of receiving 10 μ g endotoxin, the serum of intoxicated rats (but not of controls) contained a factor which regularly depressed serum Zn in normal recipient rats. This factor could be destroyed by heating serum to 90 C for 30 min. Since endotoxin is known to stimulate the release of endogenous pyrogen from PMN leukocytes, this substance was isolated from peritoneal leukocytes of rats. When administered in varying doses IP to endotoxin-tolerant rats, leukocytic pyrogen produced significant decreases in serum Zn and Fe which were linear to the log doses administered. These results indicate that the decreases observed during endotoxemia are mediated by leukocytic pyrogen. Similar results occurred when other species were tested.

Summary, Part I:

Significant decreases in serum Fe, total Fe-binding capacity, and serum Zn levels were demonstrated in humans infected with P. tularensis and attenuated VEE vaccine. Serum Fe, total Fe-binding capacity, and serum Zn values were shown to fall early in the course of infection,

and the degree of Fe and Zn reduction was found to parallel closely the degree of the febrile response. Recent animal studies have shown increasing evidence that the release of an endogenous pyrogen is involved in the reductions of serum Fe and Zn in the infected host.

Progress, Part II:

The sequential changes in blood pH, PCO_2 , PO_2 , phosphate (PO_4), pyruvate and lactate during infection have received scant attention. It has been shown that during infection accompanied by fever there is an increase in blood pyruvate and lactate^{2/} and a decrease in serum and urinary inorganic PO_4 . The hyperventilation that accompanies fever has been shown to lead to a respiratory alkalosis, which, in turn, stimulates both the conversion of inorganic PO_4 to organic PO_4 and the renal reabsorption of PO_4 , resulting in reductions in blood and urinary inorganic PO_4 . The elevated levels of pyruvate and lactate during fever have been ascribed to increased activity of the glycogenolytic and glycolytic pathways, perhaps as a result of glucocorticoid excess.

It is planned to study the sequential changes in these parameters during infection with Diplococcus pneumoniae. Preliminary results in the rat have been inconclusive, and it is planned to initiate experiments in monkeys. It is hoped eventually to follow these parameters during infection in man.

Summary, Part II:

Infection accompanied by fever causes increases in blood pH, pyruvate, and lactate and a decrease in inorganic PO_4 in blood and urine. The sequential changes in these parameters have not been established, but will be investigated.

Publication:

1. Pekarek, R. S., and W. R. Beisel. 1969. Zinc depressing effects of endotoxin and leukocytic pyrogen in the rat. Fed. Proc. 28:691. (Abstract)

LITERATURE CITED

1. U. S. Army Medical Unit. 1 July 1968. Annual Progress Report, FY 1968. p.1 to 10. Fort Detrick, Maryland.
2. Gilbert, V. E. 1968. Blood pyruvate and lactate during febrile human infections. Metabolism 17:943-951.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 002: Role of Hormones in Infectious Disease

Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland

Divisions: Physical Sciences and Medical

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Authors: John L. Winnacker, Major, MC (I)
William A. Harrison, Captain, VC (II)
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William R. Beisel, M.D. (I)

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10. NO./CODES:*		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER		
a. PRIMARY		62706A		1B662706A096		01		002		
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11. TITLE (Precede with Security Classification Code)*										
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12. SCIENTIFIC AND TECHNOLOGICAL AREAS*										
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21. GENERAL USE				ASSOCIATE INVESTIGATORS						
Foreign intelligence considered.				NAME: Woeber, K. A.						
				NAME: Harrison, W. A. DA						
22. KEYWORDS (Precede EACH with Security Classification Code)										
(U) Growth hormone; (U) Thyroid hormone; (U) Thyroxine; (U) Encephalitis, Equine (VEE); (U) Adenovirus; (U) Pneumococcal infections; (U) Salmonella; (U) Radioimmunoassay										
23. TECHNICAL OBJECTIVE* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)										
23. (U) Study the role of hormones in the response of the host to infectious disease.										
24. (U) A radioimmunoassay will be employed to study changes in the concentration of growth hormone (GH) in plasma during experimentally induced infection in human subjects. A double isotope tracer technique which will permit concurrent assessment of alterations in the rate of secretion and peripheral metabolism of thyroid hormone will be employed to study the effects of experimentally induced infection on thyroid hormone economy in the rhesus monkey. A study will be designed to assess alterations in the thyroid hormone-serum protein interaction during experimentally induced infection in human subjects.										
25. (U) 68 07 - 69 06 - Increases in plasma GH have been observed in human subjects infected with attenuated VEE virus, adenovirus, or <u>Salmonella typhosa</u> . Although the increase in plasma GH occurred generally only with febrile illness, the magnitude of increase appeared to be determined more by the type of infection than by the magnitude of fever. The significance of these findings is currently under study. Preliminary observations suggest an increase in the rate of thyroid hormone secretion during experimentally induced pneumococcal infection in the rhesus monkey.										
Publications: J. Clin. Endocr. 28:1220-1223, 1968. New Engl. J. Med. 280:541-546, 596-604, 1969. Biochim. Biophys. Acta 174:761-763, 1969.										

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 002: Role of Hormones in Infectious Disease

Description:

Study the role of hormones in the response of the host to infectious disease.

Progress, Part I:

There is considerable evidence that man responds to a variety of physical and psychological stresses with an increased secretion of adrenocorticotrophic hormone (ACTH) and growth hormone (GH), but not of other major anterior pituitary hormones. Further studies, originating primarily from this unit, have demonstrated a similar response of the anterior pituitary to the stress of acute infection and the administration of bacterial endotoxin. However, in contrast to the abundant literature on pituitary-adrenal activity during infection, only one meaningful report has been published detailing GH changes.^{1/} In order to characterize as precisely as possible the function of the hypothalamic-pituitary axis during infection, we have undertaken a systematic study of GH metabolism during various infections in man and the rhesus monkey. In this initial phase of the study, basal plasma growth hormone levels were measured in volunteers before and after exposure to Venezuelan Equine Encephalomyelitis Virus, live attenuated (VEE vaccine), type 7 adenovirus, and a virulent culture of Salmonella typhosa.

Initial efforts were directed at developing a radioimmunoassay with which to measure plasma GH. A reliable method was developed which featured both precision and sensitivity sufficient for the routine detection of GH in quantities as small as 0.03 ng (0.03 ng/ml plasma).

Five or more control values were routinely obtained on volunteers after their admission to the project wards. These specimens, as well as postinfection samples, were obtained by venipuncture at 0800 hours with subjects in a fasting and resting state. The mean 394 control values from 76 subjects was 2.3 ± 0.3 ng/ml, with over one-third of the values grouped between 0.8 and 1.2 ng/ml. The occurrence of only 16 control values in excess of 5.0 ng/ml indicated that satisfactory conditions prevailed on the project wards for the study of a hormone noted for its extremely labile secretory pattern. It was noteworthy that 8 of these 16 elevations occurred on the day of exposure, suggesting that at least in certain subjects the anticipation of infection may have been a sufficient stress to trigger an increased release of GH.

GH values were obtained for 18 consecutive days in 30 volunteers who received VEE vaccine, and in 10 uninfected volunteers who served as controls. When resulting illness was classified as asymptomatic, mild, or typical in accordance with the magnitude of the febrile response, and GH data were grouped similarly, no significant increase in GH concentration was noted in 15 asymptomatic subjects, 4 volunteers with mild early VEE illness, or in the 10 control subjects. Distinct increases did occur in 5 subjects with mild late illness (mean peak GH - 7.9 ng/ml, with corresponding fever index of 14) and in 5 volunteers with typical illness (mean peak GH - 17.1 ng/ml, with corresponding fever index of 25). The initial increase in GH concentration in these 2 groups coincided with, or preceded by up to 12 hr, the onset of fever; increased values persisted for the ensuing 4 or more days of clinical illness. It was apparent from this and subsequent studies that the factor, or factors, responsible for stimulating GH secretion in infection are probably not operative during the incubation stage of the disease.

GH concentrations were measured on 20 consecutive mornings in 23 volunteers who ingested a type 7 adenovirus vaccine. They were significantly elevated in the one subject who developed typical illness (peak GH - > 10 ng/ml, with a corresponding fever index of 20). The other 22 volunteers were classified as asymptomatic on the basis of a lack of febrile response and did not demonstrate a rise in plasma GH. Of particular interest, however, were 8 members of this group who, despite their lack of fever, did develop significant clinical illness. Thus, disabling but afebrile, acute infectious illness was not accompanied by an increase in plasma GH, whereas febrile illness was characterized by increased values. Further, peak values in volunteer groups with mild and typical illness in all studies reported occurred on days of maximum fever, and the extent of the increase in both individual patients and volunteer groups tended to parallel the febrile response. There was, however, no statistical correlation between GH concentrations at 0800 hours and the magnitude of fever during the preceding 8 hr ($r_{31} = 0.04$), suggesting that fever itself was not a stimulus to GH secretion.

Fourteen determinations of plasma GH were obtained on each of 10 volunteers infected with S. typhosa. Although the resulting illness was the most severe and protracted of the 3 infections studied, with prominent symptoms and fever persisting for a week or longer, GH concentrations were only modestly increased in 2 patients with typical illness (mean peak GH - 7.2 ng/ml, with a corresponding fever index of 37) and not at all in 3 patients with mild illness. These results were of particular interest since the GH response to Salmonella endotoxin has also been reported as small when compared to that of other bacterial endotoxins. When contrasted with the GH responses to VEE vaccine,

the data suggest that the magnitude of the GH response to various infections may be determined more by the nature of the infecting organism than by the severity of the resulting illness.

Human GH concentrations were also measured during experimental tularemia, but the values were probably inaccurate because of prolonged exposure of the specimens to room temperature. The results did indicate, however, that distinct increases in plasma GH occurred in 4 volunteers with typical illness.

Summary, Part I:

The results of 1380 basal GH determinations in 63 volunteers infected with VEE vaccine, adenovirus, and S. typhosa are presented. Increases in plasma GH occurred in subjects who developed a febrile response to these infections, whereas afebrile illness, regardless of its severity, was only rarely associated with increased values. The increase in plasma GH usually became apparent with the onset of fever, and persisted during the ensuing 3-7 days of clinical illness. There was, however, no statistical correlation between GH concentrations and the magnitude of fever during the preceding 8 hr. Although GH levels in patients exposed to the same infection tended to parallel the severity of clinical illness for that group of patients, the same observation could not be extended to patients infected with different microorganisms. Thus, VEE infection elicited far greater plasma GH concentrations than typhoid fever, although the clinical illness caused by VEE was by comparison much less severe. These preliminary results indicate that the hypothalamic-pituitary response to infection is far more complex than suggested by prior studies of pituitary-adrenal function in infection. They have also provided a direction for related studies which will hopefully increase our understanding not only of host response to infection, but also of basic mechanisms involved in hypothalamic-pituitary function.

Progress, Part II:

The role of the thyroid gland in the response of the primate host to acute infection has not been adequately assessed. The results of studies based upon measurement of the static concentration of thyroid hormone in blood during infection are difficult to interpret. Although alterations in the concentration of thyroid hormone may occur, such alterations do not necessarily imply changes in the rate of hormonal secretion, but may merely reflect changes in the thyroid hormone-serum protein interaction or changes in peripheral hormonal metabolism or both during infection. Furthermore, owing to the large extrathyroidal pool of hormone, small changes in hormonal secretion may not be

detectable by measuring the concentration of hormone in blood. Similarly, the results of studies based upon epithyroid counting following the injection of radioactively labeled inorganic iodide are open to the criticism that this technique does not discriminate between the release of radioactively labeled iodide arising from intrathyroidal deiodination and the release of radioactively labeled hormone. Consequently, a technique is presently being employed which permits concurrent assessment of alterations in the rate of secretion of thyroid hormone itself and alterations in the kinetics of peripheral hormonal metabolism. This technique which is being performed in the rhesus monkey involves the injection of 2 radioisotopes of iodine, inorganic ^{125}I to label the thyroid gland, thereby producing endogenously synthesized ^{125}I -labeled thyroid hormone, and ^{131}I -labeled thyroxine to label the peripheral hormonal pool. The concentrations in serum of endogenously labeled thyroid hormone, reflecting both hormonal secretion and metabolism, and exogenously labeled thyroxine, reflecting hormonal metabolism alone, are then measured daily before and during experimentally induced infection. Preliminary observations with this technique suggest that the rate of hormonal secretion increases remarkably during pneumococcal infection.

Summary, Part II:

A double isotope tracer technique is being employed in the rhesus monkey to study alterations in thyroid hormone economy during infection. Preliminary observations suggest an increase in the rate of thyroid hormone secretion during experimentally induced pneumococcal infection.

Progress, Part III:

The interaction of thyroxine with its binding proteins in serum conforms to a reversible binding equilibrium in which most of the hormone is bound and only an exceedingly small proportion (about 0.03%) is unbound or free. The free hormone is thought to be the metabolically active component available to the tissues. During illness, the proportion of free hormone increases greatly and this increase has been ascribed to the decrease in the binding capacity of thyroxine-binding prealbumin that accompanies illness. Recent work, however, has indicated that thyroxine-binding prealbumin binds only a small proportion of the total thyroxine in human serum and therefore cannot account for the increase in the proportion of free hormone that occurs during illness.^{2/}

It is therefore planned to study by means of reverse-flow electrophoresis the distribution of thyroxine among its binding proteins in serum collected from human subjects before, during, and after experimentally-induced infections.

Summary, Part III:

The thyroid hormone-protein interactions in serum will be studied during experimentally induced infection in human subjects.

Presentations:

1. Winnacker, J. L., W. R. Beisel, and M. I. Rapoport. Growth hormone responses to acute infection. Presented at The Endocrine Society, 27-29 June 1969, New York, N. Y.

Publications:

1. Beisel, W. R., K. A. Woeber, P. J. Bartelloni, and S. H. Ingbar. 1968. Growth hormone response during sandfly fever. J. Clin. Endocr. 28: 1220-1223.

2. Beisel, W. R., and M. I. Rapoport. 1969. Interrelationships between adrenocortical functions and infectious illness. New Engl. J. Med. 280: 541-546, 596-604.

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1. Beisel, W. R., J. Bruton, K. D. Anderson, and W. D. Sawyer. 1967. Adrenocortical responses during tularemia in humans. J. Clin. Endocr. 27: 61-69.

2. Woeber, K. A., and S.H. Ingbar. 1968. The contribution of thyroxine-binding prealbumin to the binding of thyroxine in human serum, as assessed by immunoadsorption. J. Clin. Invest. 47:1710-1721.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 003: Tissue Enzyme Changes in Infectious Disease

Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland

Division: Physical Sciences and Medical

Period Covered by Report: 1 July 1968 to 30 June 1969

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Reports Control Symbol: RCS-MEDDH-288 (R1)

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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
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				DA			
22. KEYWORDS (Precede each with Security Classification Code) ^a (U) Tryptophan; (U) Tyrosine; (U) Tolerance tests; (U) Typhoid fever; (U) Tularemia; (U) Sandfly fever; (U) Rocky Mountain spotted fever; (U) Enzyme induction							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23. (U) To study serial changes in tissue enzyme systems during the course of experimental infections. 24. (U) Tryptophan tolerance tests are performed serially in individuals in whom infections have been experimentally induced, in order to study the activity of hepatic tryptophan pyrrolase (TP). Urinary diazo reactants and kynurenine pathway metabolites are measured. Since maternal and fetal tyrosine transaminase (TT) activities are sensitive to different stimuli, the pregnant female rat is used as a model system to separate different factors responsible for the rise in TT activity during infection. 25. (U) 68 07 - 69 06 - During typical acute typhoid fever, the excretion of diazo reactants and some kynurenine pathway metabolites after a tryptophan load was greatly increased over control or incubation period values. Similar changes, though of smaller magnitude, were observed during other infections. Changes were rarely seen during the incubation period and usually reverted to normal early in convalescence. Diurnal periodicity of the kynurenine pathway persisted during infection. Preliminary studies have indicated the suitability of using the pregnant female rat as a model to study factors that control TT activity during infection. Extensive studies using oral tyrosine tolerance tests in man indicated that the test results and diurnal rhythmicity of this amino acid are easily influenced by the timing and content of dietary intake but not by steroid administration or bacterial infection.							

^aAvailable to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 003: Tissue Enzyme Changes in Infectious Diseases

Description:

To study serial changes in tissue enzyme systems during the course of experimental infections.

Progress, Part I:

Previous work was continued concerning serial changes in hepatic tryptophan pyrrolase (TP) activity during induced infections in man. This work was a joint effort with Drs. Morton I. Rapoport and Richard B. Hornick, Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland (Contract DA49-193-MD-2867). Earlier studies in experimental animals demonstrated induction of hepatic TP during acute infectious illness.^{1,2/} Since this enzyme had a circadian periodicity in animals and since a circadian periodicity of tryptophan metabolism could be demonstrated in man, it was reasoned that altered enzyme activity could be studied in man by an indirect means, i.e. by measuring tryptophan metabolism after a 3 gm oral tryptophan load. Complete serial studies have now been accomplished in a total of 40 volunteers in studies of tularemia and sandfly fever at USAMRIID and typhoid fever and Rocky Mountain spotted fever (RMSF) at the University of Maryland.

With the exception of sandfly fever, a mild self-limited illness, all patients developing symptoms of induced infection were treated promptly with the appropriate antibiotic; no complications were encountered. Patients with tularemia and sandfly fever generally developed illness within 3-4 days. Their total period of fever and symptoms was seldom more than 3 days. On the other hand, typhoid fever was characterized by a longer, less predictable duration of incubation, 7-20 days in length. The subsequent illness in this study was of low toxicity although patients were symptomatic for approximately 5-7 days. A live attenuated vaccine strain was used in tularemia studies; in the typhoid fever studies, a monovalent, enteric coated, acetone-killed and dried oral vaccine of the Fy 2 strain was used in a dose of 2 tablets per day for 3 days.

In the RMSF studies, a commercial killed vaccine (Lederle Laboratories) was employed in 3 weekly 1-ml doses. The volunteers were inoculated 4-6 months later by the intradermal administration of 10 median guinea pig intraperitoneal infectious doses (GPIPID₅₀) of

Sheila Smith strain of Rickettsia rickettsi. Incubation periods ranging from 6-10 days were followed by fever, headache, and myalgia, with an exanthem of the trunk and extremities appearing on the 2nd or 3rd day of fever. Symptomatic illness responded promptly to chloramphenicol, doxycycline, or tetracycline therapy.

Each individual was studied with a series of tryptophan tolerance tests which included control and postexposure measurements. These tests were conducted at 1800 hours, a time of day when normal functional activity of the kynurenine pathway is low. In addition, diurnal influences were studied by dividing the RMSF volunteers into 2 groups for testing at 0600 or 1800 hours.

In general, studies during incubation failed to reveal any appreciable change from control period observations. Occasional subjects in the typhoid and RMSF groups did show an increased excretion of total diazo reactants, kynurenine, kynurenic acid and/or 3-OH-kynurenine. In several instances these individual changes were reflected by significant increases in the group average.

No consistent variation or trend was noted in values of the subjects who remained asymptomatic after exposure to Pasteurella tularensis; this suggested that test values were not altered systematically by the administration of 3.0 gm tryptophan on 4 consecutive days. No apparent change was noted in the 2 sham-exposed subjects in the sandfly fever study.

The excretion of diazo reactants and kynurenine pathway metabolites during illness was consistently increased above control or incubation period values when tolerance tests were performed in the morning. These increases were greatest, by far, in subjects with typical typhoid fever; intermediate in those with mild typhoid fever, sandfly fever, and RMSF; and least in those with symptomatic tularemia. Average values became higher as illness progressed in the typical typhoid fever group. Because tryptophan tolerance tests were conducted only during days of incubation or early symptoms in subjects exposed to P. tularensis, the time of maximal increases in diazo excretion may have been missed in more than half of the symptomatic individuals. This fact, along with the relative mildness of illness in other members of the symptomatic tularemia group may account for the relatively small increase in their excretion of diazo reactants. These data, in combination, suggested that increased diazo excretion was not related directly to the etiology of infection but seemed instead to increase proportionally in magnitude to the severity of illness. Such a correlation was highly significant ($p < 0.001$) for diazo excretion. Because of the small number of subjects and the relatively homogeneous clinical response within each group, significant correlations with fever were not evident for individual diseases.

When the individual excretory products of tryptophan were analyzed in a manner similar to that recorded above, kynurenine and xanthurenic acid excretion were each found to correlate with fever with a significance equivalent to that of the total diazo reactants. Correlation with fever was good when 3-OH-kynurenine was considered ($p < 0.01$), minimal with o-amino hippuric acid excretion, and non-significant with respect to kynurenic acid or anthranilic acid glucuronide excretion.

Analysis of simultaneously excreted products of tryptophan metabolism in each tolerance test allowed an examination of the possibility that branches of the kynurenine pathway might be influenced differently by infectious illness or by specific etiologies of infection. A 2- to 4-fold increase of all metabolites was detected most frequently. If data from typical typhoid fever patients were omitted, a lack of clearcut differences in the magnitude of increment among the individual metabolites suggested that the increased metabolism of tryptophan involved all kynurenine pathways. Such an observation could result from an increased activity of TP. Consideration of the disproportionately large increases of kynurenine, xanthurenic acid and o-aminohippuric acid excretion in subjects with typical typhoid fever suggested, in addition, that a greater utilization of the pathway (in the face of a greater and more protracted illness) had allowed several more distal branch pathway enzymes to become rate-limiting.

Further information was gained by comparing the results of tolerance tests conducted in the morning and evening. The course of RMSF was of equivalent clinical severity in both groups so that evaluation of these data was not obscured by differences in either the etiology or severity of illness. There was an increase above morning control values in the urinary excretion of total diazo reactants and all kynurenine pathway metabolites with the exception of kynurenic acid. While an illness-related increase was typical of both morning and evening tests, these data make it clear that excretion during the latter test period of kynurenine pathway metabolites at the height of illness was generally below the control period values for the former, and therefore far below them during illness. Thus, diurnal periodicity of this pathway was found to continue during the course of infectious illness even though it was modified by the generally greater activity associated with fever and symptoms.

During convalescence, loading tests were characterized by reduction in excretion of kynurenine pathway metabolites to or toward values observed during the control period.

Summary, Part I:

Augmented urinary excretion of diazo reactants during infections in man is thought to reflect an altered metabolism of tryptophan. Correlation between excretion of tryptophan metabolites and activity of hepatic TP has been established. To detail this relationship and its significance during infection, tryptophan loading tests were performed serially in individuals in whom typhoid fever, RMSF, tularemia, and sandfly fever had been experimentally induced. These investigations were performed ancillary to well controlled evaluations of vaccine efficacy. Following the oral administration of 3 gm of tryptophan, urinary diazo reactants and individual kynurenine pathway metabolites were measured. Typical acute typhoid fever produced 8- to 10-fold increases in diazo reactants and the majority of pathway metabolites. Similar changes of smaller magnitude were observed during mild typhoid fever and in the other infections. Kynurenine pathway augmentation appeared to parallel the severity of illness. Rarely did changes develop during the incubation period, and reversion to normal values generally were observed early in convalescence. Utilization of this pathway appeared to maintain a diurnal rhythmicity despite the presence of acute infectious illness.

Progress, Part II:

Previous studies from this unit have demonstrated that tyrosine transaminase (TT) activity in the liver of the rat has a circadian rhythm and that during experimentally produced pneumococcal infection TT activity in the liver is increased.^{1,2} As both the circadian rhythm and the rise following infection were abolished in adrenalectomized rats it has been suggested that the changes observed were controlled by the amount of glucocorticoid hormone secreted. However, because of the complexity of the metabolic, endocrine, and biochemical responses during infection, additional important control mechanisms may have been overlooked, especially if their effects depend on the presence of glucocorticoid hormones. In order to overcome this difficulty, the effect of experimentally produced pneumococcal infection on hepatic TT activity in pregnant female rats and their fetuses will be studied. The liver of the fetal rat appears unique in that glucocorticoid hormones do not increase the activity of TT. However, fetal TT can be increased by a number of other hormones and small molecules which are known to increase TT activity in the liver of the adult rat. Thus, if hepatic TT activity of the fetus were to be increased during experimentally produced pneumococcal infection in the mother it would be due to factors other than the increase in glucocorticoid hormone secretion. An additional advantage of this model is that liver tissue from the fetal rat can be cultured easily. Thus, it is possible to study the effects observed in vivo in a precisely controlled in vitro system.

Preliminary studies have been done to determine the activity of TT in the 100,000 x g supernatant fraction from livers of 15-, 17-, and 20-day old pregnant rats and their fetuses. Maternal TT activity was found to be approximately 25 times greater than fetal TT activity. In addition the maternal TT activity was found to be greater at 2200 than at 1000 hours which is in agreement with the findings of Shambaugh et al.^{3/} The fetal TT activity showed no evidence of circadian periodicity, which is in agreement with published findings that fetal TT activity is not stimulated by glucocorticoid hormones in vivo. Organ cultures of fetal liver tissue have been maintained for periods up to 48 hr and preliminary experiments have been carried out to determine the ability of varying concentrations of hydrocortisone to increase the TT activity of isolated fetal liver explants.

Summary, Part II:

Preliminary studies have been carried out and confirm the suitability of using the pregnant female rat as a model to study the factors that control TT activity during experimentally induced pneumococcal infection.

Progress, Part III:

Oral tyrosine tolerance tests were employed to study tyrosine metabolism in healthy and infected volunteers (Medical Division Project FY 68-6). The selection of this procedure was prompted by experience with a tryptophan tolerance test which demonstrated a characteristic diurnal variation in tryptophan metabolism in normal subjects, and distinct changes during infection. The tyrosine tolerance test was performed by measuring tyrosine in heparinized plasma obtained prior to, and 0.5, 1.5, 4.5 and 6 hr following the administration of 3.5 gm of L-tyrosine to fasting subjects.

The following studies were obtained on 12 healthy, hospitalized volunteers: (1) Tyrosine tolerance tests in different subjects beginning at 4 hour intervals throughout the day; (2) Tolerance tests at 0400 and 0800 hours in subjects who had ingested 2.0 mg of dexamethasone for 2 days; and (3) Tolerance tests at 0400 and 0800 hours simultaneously with the ingestion of breakfast. Hourly plasma samples were obtained throughout the day to determine normal plasma tyrosine periodicity. Although initial studies had suggested differences between tyrosine tolerance values obtained during the 0400- and 0800-hour tests, there was no evidence in the completed study that the time of tyrosine administration significantly altered tyrosine tolerance.

Tests performed after steroid administration at both 0400 and 0800 hours revealed tyrosine values which were virtually identical with control values. The primary effect of eating a regular meal at the time of the tyrosine loading was to elevate the values obtained during the last 3 hr of the test. The initial curve was not affected. Plasma collected with volunteers in a fasting state when tyrosine was withheld revealed significantly higher tyrosine concentrations between 2400 and 1000 hours than during the rest of the day, a result contrary to that obtained at other times in this and other laboratories.

Tyrosine tolerance tests were also performed on 8 volunteers exposed to Pasteurella tularensis (2 who developed typical illness, 3 with mild illness, and 3 with asymptomatic responses). Each volunteer underwent 4 tests at 0800 hours: (1) one day prior to exposure, (2) one day after exposure, (3) during clinical illness, and (4) during convalescence. There was no evidence from these studies of a consistent effect of infection on the normal pattern of tyrosine tolerance. These studies, in toto suggest that: (1) Diurnal variation in plasma tyrosine is probably strongly influenced by both the timing and content of prior food intake, a factor not adequately evaluated in our studies; (2) The simultaneous ingestion of food and tyrosine significantly affects subsequent plasma tyrosine tolerance test values; (3) Tyrosine tolerance at 0400 and 0800 hours is not altered by pretreatment with corticosteroids in doses sufficient to abolish the normal circadian rhythm of adrenal steroids (providing indirect evidence that the corticosteroid-induced enzyme, tyrosine transaminase, is probably not a major factor determining the pattern of tyrosine tolerance); and (4) Acute bacterial infection does not consistently alter tyrosine tolerance in man.

Summary, Part III:

Extensive studies using oral tolerance tests in man indicate that the test results and diurnal rhythmicity of this amino acid are easily influenced by the timing and content of dietary intake, but not by steroid administration or bacterial infection.

Presentation:

1. Rapoport, M. I., W. R. Beisel, R. B. Hornick, M. J. Snyder, and B. Crevey. Altered tryptophan metabolism in Salmonella infections, presented at Federation of American Societies for Experimental Biology, Atlantic City, N. J. 13-18 April 1969.

Publications:

None

LITERATURE CITED

1. U. S. Army Medical Unit. 1 July 1967. Annual Progress Report, FY 1967. p. 11 to 16. Fort Detrick, Md.
2. U. S. Army Medical Unit. 1 July 1968. Annual Progress Report, FY 1968. p. 17 to 22. Fort Detrick, Md.
3. Shambaugh III, G. E., D. A. Warner, and W. R. Beisel. 1967. Hormonal factors altering rhythmicity of tyrosine-alpha ketoglutarate transaminase in the liver. Endocrinology 81: 811-818.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 005: Evaluation of Normal Colony Animals

Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland

Divisions: Animal Assessment and Pathology

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10. NO./CODES*		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
						WORK UNIT NUMBER	
a. PRIMARY		62706A		1B662706A096		01	
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12. SCIENTIFIC AND TECHNOLOGICAL AREAS*							
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17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
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b. NUMBER:*				FISCAL		69	
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19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence considered.				NAME: Weil, J. D.			
				NAME: Trevino, G. S. DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Animals, laboratory; (U) Enteric infection; (U) Tuberculosis; (U) Hematology; chemistry, blood; (U) Necropsy							
23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Obtain clinical and pathological baseline values. Establish patterns of disease in normal colony animals.							
24. (U) Conduct studies on colony animals to establish normal values for various biological parameters of interest to investigators using animals as test subjects. Study incidence and patterns of disease in the animals.							
25. (U) 68 07 - 69 06 - Baseline hemograms and blood chemistry values were determined for 1122 bleedings of 374 monkeys acquired during the year. Fecal examinations of these monkeys revealed a 45% incidence of nonpathogenic protozoan cysts and a 15% incidence of nematode species. Reactions to tuberculin were negative; seropositivity to staphylococcal enterotoxin B was found in 12.8%. Gastroenteritis was the most common disease problem. A screening study for enteric pathogens was conducted on 40 conditioned monkeys. <u>Shigella flexneri</u> was isolated from 38%.							
Contagious ovine ecthyma was diagnosed clinically and serologically in 6 goats.							
Four monkeys, 6 dogs and 1 horse that died were necropsied with varied findings.							

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 005: Evaluation of Normal Colony Animals

Description:

Obtain clinical pathological baseline values and establish patterns of disease in normal colony animals.

Progress, Part I:

During the past year, we have continued to collect data from the colony animals on normal blood values, parasitic infestations and disease patterns.

Monkeys. - The following table represents hematologic data on 374 rhesus monkeys (Macaca mulatta) received during FY 1969. These data were obtained and analyzed using the same procedures and techniques as reported previously.^{1/} In investigating sex-related differences, a higher percentage of neutrophils was found in females maintained in a mixed population of monkeys as compared to females in an entirely female population. Further studies are in progress to determine if this is hormonal or environmental.

Limited data are available on total protein, albumin and albumin/globulin ratio.

Qualitative fecal examinations using the ether-formalin concentration method showed a 45% incidence of nonpathogenic protozoan cysts, the majority being Entamoeba coli. It was also found that 15% had some type of nematode ovum, but 1 group had a 76% incidence, accounting for much of the 15%. Oesophagostomum and Strongyloides were the 2 most common parasites. Three fluke ova were detected during the year.

During the year, no positive reactors were detected from 1,666 intrapalpebral tuberculin tests; 12.8% of the monkeys received were serologically positive for staphylococcal enterotoxin B (SEB), as compared to 9.3% during FY 1968; and approximately 400 monkeys were smallpox-vaccinated.

Gastrointestinal infection continued to be the main disease in the colony monkeys with an overall incidence of 57%. One-third of these had hemorrhagic gastroenteritis with Shigella flexneri 4 being isolated from nearly half the hemorrhagic cases; the same species was isolated from only 3 nonhemorrhagic

cases. Surveys in our colony have shown that a significant percentage of monkeys carry this bacterial pathogen asymptotically.

TABLE I. RESULTS OF 1122 BLEEDINGS ON 374 MEDICAL RESEARCH INSTITUTE RHESUS MONKEYS.

VARIABLE	UNITS	MEAN \pm 1 SD
Leukocytes	no./mm ³	10,732 \pm 3,000
Neutrophils	no./mm ³	3,820 \pm 1,700
Lymphocytes	no./mm ³	6,610 \pm 2,100
Monocytes	no./mm ³	112 \pm 166
Eosinophils	no./mm ³	191 \pm 261
Basophils	no./mm ³	23 \pm 49
Packed cell volume	%	40.7 \pm 2.5
Hemoglobin	gm/100 ml	13.3 \pm 0.9
Blood urea nitrogen	mg/100 ml	18.0 \pm 3.5
Serum transaminase glutamic oxalacetic	Sigma-Frankel	34.5 \pm 18.0
glutamic pyruvic	Sigma-Frankel	30.0 \pm 10.2
Alkaline phosphatase	Bodansky	18.6 \pm 6.6

Weight gain studies indicate decreased rate of gain during the first 30 days of colony residence, presumably due to enteritis.

Equines and Canines. - Each animal was screened at least once during the past year for blood values and several times, as indicated, for parasites. Strongylus sp. continued to be common in the ponies and burros while Toxocara canis was a problem in several litters of puppies.

Goats. - Contagious ovine ecthyma (CE) was diagnosed clinically in 6 goats acquired from a local supplier; serologic tests and viral isolation have been done to confirm the diagnosis. The goat with the most severe clinical disease developed a 1:64 complement fixation titer to CE.

Summary, Part I:

Hematologic data is presented on USAMRIID monkeys. Gastroenteritis remains the most common disease in the monkey colony. Screening for parasites revealed similar conclusions as FY 1968. Contagious ovine ecthyma was diagnosed clinically and confirmed serologically in the goats.

Progress, Part II:

Rectal swabs from 40 rhesus monkeys (M. mulatta) were cultured over a 3-mon period from 11 Nov 68 - 27 Jan 69. Prior to this time, these monkeys had been conditioned a minimum of 3 mon at the Fort Detrick animal farm and a minimum of 30 days at the USAMRIID animal holding area. Rectal swabs were obtained twice weekly regardless of the health of the animal.

Rectal swabs were inoculated directly onto EMB and xylose-lysine-deoxycholate agar plates. The swabs were then placed in selenite broth for overnight incubation, which was then streaked onto SS agar. Selenite broth was eliminated midway through the project and swabs were inoculated directly onto SS agar.

Colonies were identified and recorded with special attention given to possible Salmonella and Shigella types. Suspect colonies were selected and transferred to Kligler's iron agar (KIA), and all colonies producing H₂S were transferred from KIA to urea agar for elimination of Proteus. No Salmonella were isolated.

Organisms with typical Shigella reactions on KIA were inoculated into indol, methyl red, Voges-Proskauer, citrate, motility and KCN media. Suspect organisms were then serotyped with Shigella antisera and confirmed with further biochemical studies.

Suspicious organisms that had a positive KCN reaction were retained for further identification. Identification is still in progress.

To date, S. flexneri 4 has been identified from 15 of the 40 monkeys. Of these 15, 12 were clinically healthy at the time of recovery of Shigella, while 3 were from monkeys with enteritis.

Summary, Part II:

A screening study for enteric pathogens was conducted on 40 conditioned monkeys over a 3-mon period. Fifteen of the 40 animals had one or more isolations of S. flexneri 4 during this period, 3 of which were clinically affected with enteritis at the time of culture.

Progress and Summary, Part III:

Four monkeys, 6 dogs, and 1 horse were necropsied during the past fiscal year. Table II summarizes the findings.

TABLE II. CAUSES AND DISTRIBUTION OF 11 COLONY ANIMAL DEATHS.

ANIMAL	CAUSE	DISTRIBUTION OF CASES
Horse	Equine infectious anemia (presumptive)	1
Monkey	Aborted fetus, etiology unknown	1
	Acute gastric dilatation	2
	Enteritis, bacterial	1
Dog	Traumatic fracture of spine with compression of spinal cord	1
	Ascariasis, with occlusion of bile duct	1
	Undetermined (newborn pups)	4

Publications:

None.

LITERATURE CITED

1. U. S. Army Medical Unit. 1 July 1968. Annual Progress Report, FY 1968. p. 29 to 33. Fort Detrick, Maryland.

ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 008: Cellular Changes During the Immune Response

Reporting Installation: U.S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland

Division: Bacteriology

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Authors: Robert E. Krisch, Captain, MC
Martha K. Ward, Captain, USPHS

Reports Control Symbol: RCS-MEDDH-288(R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OLO869	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMRY ^a	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DISSEM INSTR ^a	9. SPECIFIC DATA- CONTRACTOR ACCESS	10. LEVEL OF SUM A. WORK UNIT
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES: ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62706A		1B662706A096		01	
b. CONTRIBUTING		62124011		1B622401A096		01	
c. CONTRIBUTING		CDOG 1212b(9)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) Cellular changes during the immune response							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
003500 Clinical Medicine; 004900 Defense; 003200 BW, CW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
66 08		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PREVIOUS		b. FUNDS (in thousands)	
b. NUMBER: ^a				FISCAL		69	
c. TYPE:				YEAR		70	
d. KIND OF AWARD:				CURRENT		0	
19. ESTIMATED COMPLETION DATE				20. PERFORMING ORGANIZATION			
NAME: ^a USA Medical Research Institute of Infectious Diseases ADDRESS: ^a Fort Detrick, Md 21701				NAME: ^a Bacteriology Division USA Medical Research Institute of Infectious Diseases Fort Detrick, Md 21701 PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
RESPONSIBLE INDIVIDUAL				NAME: ^a Ward, M. K.			
NAME: Crozier, D.				TELEPHONE: 301 663-4111 Ext 3246			
TELEPHONE: 301 663-4111 Ext 5233				SOCIAL SECURITY ACCOUNT NUMBER:			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence considered.				NAME: Krisch, R. E.			
				NAME:		DA	
22. KEYWORDS (Precede EACH with Security Classification Code) ^a							
(U) Immunity; (U) Immunology; (U) RNA; (U) DNA; (U) Antibody formation							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Investigate biochemical changes in individual antibody-producing cells during the immune response, with emphasis on quantitative and qualitative changes in nucleic acid metabolism.							
24. (U) Suspensions of lymphoid cells are prepared from immunized animals. Specific antibody-producing cells are identified by means of the antibody plaque technique. DNA, RNA, and protein synthesis are then studied by means of autoradiography.							
25. (U) 68 07 - 69 06 - The principal investigator completed his tour of duty at the end of the last fiscal year, and no adequate replacement was available for continuation of these studies during this fiscal year.							
Publication: Nature 1969. In press.							

^aAvailable to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 008: Cellular Changes During the Immune Response

Description:

Investigate biochemical changes in individual antibody-producing cells during the immune response, with emphasis on quantitative and qualitative changes in nucleic acid metabolism.

Progress and Summary:

The principal investigator completed his tour of duty at the end of the last fiscal year, and no adequate replacement was available for continuation of these studies during this fiscal year.

Publications:

1. Krisch, R. 1969. DNA synthesis by antibody-forming cells during the primary immune response. Nature. In press.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 009: Amino Acid and Protein Changes in Blood in
Infectious Disease or in Conditions Induced
by Other Variables

Report Installation: U. S. Army Medical Research Institute of Infectious
Diseases
Fort Detrick, Maryland

Divisions: Physical Science and Medical

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Authors: George A. Burghen, Major, MC
Albert S. Klainer, MD
William R. Beisel, MD

Reports Control Symbol: RCS-MEDDH-288(R1)

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a DA OL0879	2. DATE OF SUMMARY ^a 69 07 01	REPORT CONTROL SYMBOL DD-R&E (AR) 636	
3. DATE PREV SUMRY 68 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY ^a U	6. WORK SECURITY ^a U	7. REGRADING ^a NA	8. DES'N INSTR'N DE	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES: ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62706A		1B662706A096		01	
b. Secondary		62124011		1B622401A096		01	
c. Secondary		CDOG 1212b(9)					
11. TITLE (Precede with Security Classification Code) ^a (U) Amino acid and protein changes in blood in infectious disease							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a 003500 Clinical Medicine; 004900 Defense; 003200 BW, CW, RW							
13. START DATE 65 07		14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. FUNDS (in thousands)	
a. DATES/EFFECTIVE:				PRECEDING		a. PROFESSIONAL MAN YRS	
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d. KIND OF AWARD:				70		3	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a USA Medical Research Institute of Infectious Diseases ADDRESS: ^a Fort Detrick, Md 21701				NAME: ^a Physical Sciences & Medical Divisions USA Medical Research Institute of Infectious Diseases Fort Detrick, Md 21701			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Crozier, D.				NAME: ^a Burghen, G. A.			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered.				ASSOCIATE INVESTIGATORS			
				NAME: Beisel, W. R.			
				NAME:			
22. KEY WORDS (Precede EACH with Security Classification Code) ^a (U) Amino acids; (U) Yellow fever; (U) Encephalitis, equine (VEE, EEE, WEE); (U) Hepatitis; (U) Liver disease; (U) Glycoproteins; (U) Lipoproteins							
23. TECHNICAL OBJECTIVE: ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23. (U) Study changes in amino acids and proteins in blood in infectious disease or in conditions induced by other variables.							
24. (U) A qualitative technique modified for the semiquantitative analysis of amino acids from small whole blood samples is applied to samples obtained from subjects with a variety of experimentally induced infections. A rapid quantitative electrophoretic method is used to measure serum proteins in plasma or serum.							
25. (U) 68 07 - 69 06 - Studies to determine whole blood amino acid changes during the course of infection with various microorganisms in man have been continued and previously reported data have been more thoroughly analyzed. Yellow fever vaccination resulted in disturbances of normal amino acid periodicity which occurred in the absence of clinical symptoms. In volunteers administered VEE vaccine, neither the circadian periodicity nor the concentration of the amino acid proline was altered. Chloramphenicol, an antibiotic which has been shown to readily inhibit protein synthesis in microbial systems but to have little effect in most mammalian systems, was shown to produce statistically significant changes in whole blood amino acids in humans.							
Prior studies on serum proteins were reported under Work Unit 096 01 001. A rapid quantitative electrophoretic method for simultaneously measuring serum proteins, glycoproteins, and lipoproteins has been developed. Some of the changes that occur as a result of infection appear to be sufficiently specific to warrant their consideration as diagnostic indicators.							
Publications: Metabolism 17:764-775, 1968; Amer. J. Clin. Path. 50:137-141, 1968. Nature 221:94-95, 1969; Arch Intern. Med., In press.							

^a Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 009: Amino Acid and Protein Changes in Blood in Infectious Disease or Conditions Induced by Other Variables

Description:

To study changes in amino acids and proteins in blood in infectious disease or induced by other variables.

Progress, Part I:

Studies to determine whole blood amino acid changes during the course of infection with various microorganisms in man have been continued, and previously reported data^{1,2/} have been more thoroughly analyzed.

One of the amino acid standards used was described last year^{2/}; use of such a mixed amino acid standard has continued, with the current distribution of acids as follows:

	<u>mg/100 ml</u>
Lycine monohydrochloride	34
Glycine	25
Glutamic Acid	15
Alanine	15
α -Amino Butyric Acid	11
Valine	11
Leucine	12

The other standard formulated in this laboratory consists solely of proline at a concentration of 10.8 mg/100 ml.

Reexamination was carried out on results from previous studies involving amino acid determinations.

Yellow Fever Vaccination with 17D Strain (Medical Division Projects FY 67-4, 67-5, 68-1). With the use of improved standards some correction was required for results presented before.^{1,2/} The 18 immunized men and 1 of 4 controls exhibited altered diurnal rhythmicity within 12-48 hr of injection. It had been stated previously that this change was apparent within 30 min.

When men were immunized at 0800 hr rhythmicity was affected in 24-48 hr; when injection occurred at 2000 hr, the affect was apparent ≤ 24 hr. The reversal lasted for 5-10 days; it had been said before that the duration was "for as long as 5 days. In the 3rd study (FY 68-1) only occasional alterations of amino acid rhythmicity were noted in 7 of 12 immunized men.

Venezuelan Equine Encephalomyelitis Virus Vaccine, Live, Attenuated, Lot 6, Immunization (VEE Vaccine) (Medical Division Project FY 68-8). It has been postulated^{3/} that there was VEE vaccine-induced inhibition of the enzyme glutamine synthetase in man. Because of the potential value of this postulation, the study was repeated and reported in part last year.^{3/} Results were considered inconclusive because of a secondary infection in some of the volunteers involved. The study was repeated (Medical Division Project FY 69-1). The frozen stored sera are to be tested with greater detail and precision for amino acids using the recently acquired Amino Acid Analyzer. Reevaluation from the standpoint of trace metals and other parameters of the earlier study indicate that only the later portion of the time of the study was affected by the inter-current infection. Final analysis of both of these projects is incomplete.

Chloramphenicol (Medical Division Project FY 68-7)^{4/} as part of a study to compare blood levels and urinary excretion of chloramphenicol given as Chloromycetin^R or an Amphicol^R, whole blood amino acids were measured.

Chloramphenicol, which has been shown to inhibit protein synthesis in microbial systems but to have little effect in most mammalian systems^{5/}, was shown to produce statistically significant changes ($p < 0.05 - 0.001$) in whole blood amino acids in humans. The most striking changes were a decrease in the single amino acids alanine and proline and a decrease in 2 amino acid groups, glycine-asparagine-serine and glutamic acid-theonine. Cystine was elevated early in the study but rapidly became normal. Other single amino acids and groups of amino acids, including glutamine, α -amino butyric, arginine-lysine-histidine, methionine-valine-tryptophan, and isoleucine-leucine, either remained unchanged or were variable.

Summary, Part I:

Studies on amino acids revealed the following: (1) 17-D strain yellow fever vaccination resulted in disturbances of normal amino acid circadian periodicity which occurred in the absence of clinical symptoms; (2) neither the circadian periodicity nor the concentration of the amino acid proline was altered by VEE vaccine, as had been reported previously; and (3) chloramphenicol produced statistically significant changes in whole blood amino acids in humans.

Progress, Part II:

Changes in serum proteins following infection have been studied for some time. Early impetus for this was the hope that patterns diagnostic for specific infectious diseases would emerge. What did result, however, was a constellation of changes which probably reflected nonspecific responses to infection or inflammation. Acute infection is characterized by an increase in the α -globulins and a decrease in albumin and chronic infection, by a decrease in albumin and

an increase in γ -globulin. The study of serum glycoproteins during infection has been hampered by the lack of a practical laboratory method. The basis for the infection-induced glycoprotein changes to be described below was the development in this laboratory of a rapid, practical, and reproducible method for serum glycoprotein determination on cleared cellulose acetate strips.^{6/} This method now allows serum glycoprotein determination with the ease usually accorded routine serum protein analysis and has eliminated the need for concentration of biologic specimens and the problem of color fading. These changes have been measured in our laboratory in a number of bacterial, viral, and protozoan infections, some of which have been previously reported.^{7-9/} This work has been expanded and now includes the study of infection-induced changes in serum protein and lipoprotein electrophoretic patterns. Recently, applying the suggestions of Kohn,^{10/} a rapid quantitative electrophoretic method for the measurement of serum and plasma lipoproteins was developed in this laboratory.

Utilizing cellulose acetate strips as the support median and barbital as the buffer (pH 8.6; ionic strength 0.05) electrophoresis is performed for 45 min at 240 volts with a current of 2.5 ma per 1" x 6" strip. Forty microliters of plasma or serum is applied to each strip by 4 applications to the same point using a wire applicator. Following electrophoresis the lipoproteins are fixed with 7.5% trichloroacetic acid to prevent blurring or smudging of the bands during the staining and washing procedures. In preparation for ozonization, the strips are hung on a circular rack in a 5-L desiccator. A 250-ml beaker containing 6 gm of BaO_2 is placed in the center of the desiccator. An acid resistant rubber tube is inserted through the exhaust hole in the lid and 25 ml of concentrated H_2SO_4 is injected into the beaker. The tube is quickly withdrawn and the exhaust valve is sealed. Ozone is generated by the reaction of the acid with the BaO_2 . Following a 10-min period of ozonization, the strips are placed in a reducing solution of 0.001 N HCl; they are then stained with Schiff's reagent for 10 min. The strips are washed with 5% acetic acid and 0.1N HCl, dehydrated with absolute methanol and cleared with 15% acetic acid in methanol. The strips are spread on a glass plate for drying and clearing which occurs in 5-10 min at room temperature. The 3 lipoprotein bands (α , pre- β , and β) are quantitated by densitometry with the results being reported as percentage of the total sample.

Since the serum protein and glycoprotein methods used in this laboratory also utilize cellulose acetate strips, the development of this method now allows a simultaneous comparison of serum protein, glycoprotein, and lipoprotein electrophoretic patterns from a single sample of serum. At the present time, we are studying factors such as diet and storage which affect serum and plasma lipoprotein electrophoretic patterns. We plan to apply this method to the study of infectious diseases.

The following infection-induced serum protein and glycoprotein changes in humans and animals have been observed:

VEE Vaccine. The data from 2 separate but very similar studies designed to determine the host response in healthy male volunteers to the administration of VEE Vaccine (FY 68-8 and FY 69-1) vaccine were combined and statistically

analyzed. Forty volunteers from the two studies were divided into four groups and their illness classified as follows: (1) Typical, if the fever hr (defined as degrees of rectal temperature > 100 F multiplied by the number of hr of fever) were > 60 or if the temperature reached > 102.5 F on any given day, (2) Mild, if the fever hr were 5-59, (3) Asymptomatic, if the fever hr were < 5 , and (4) Controls. Using this classification, there were 6 typical, 9 mild, 15 asymptomatic, and 10 control subjects.

Statistical analysis of daily serum protein and glycoprotein electrophoretic patterns revealed no significant changes in the mild, asymptomatic, or control group. Glycoprotein changes in the typical group were characterized by a slight increase in α_1 and α_2 glycoproteins and a slight decrease in the β -glycoprotein. The albumin and γ -glycoprotein fractions remained stable as did the other serum protein fractions.

Idiopathic Hemorrhagic Syndrome (IHS). The serum protein electrophoretic patterns of 13 dogs with IHS of unknown etiology were characterized by an increase in γ -globulin and a decrease in α_1 -globulin and albumin; the β - and α_2 -globulin fractions were usually normal but in a few cases were increased. Serum glycoprotein determinations revealed an increase in γ -glycoprotein and a decrease in the α_1 -glycoprotein and albumin fractions.

In one dog (King) where serial blood samples were obtained, there was a mean 300% increase in γ -globulin over the mean control value, but only a 40% increase in the mean γ -glycoprotein. This difference between the concentrations of γ -globulin and γ -glycoprotein may indicate that the γ -globulin produced in this dog was low in carbohydrate, e.g. 7S γ -globulin contains 2.8% carbohydrate compared to 11.1% for 19S γ -globulin. The increase γ -globulin levels suggest that the disease was diagnosed after the acute immunologic response had occurred.

Thirty-six additional samples of serum have been obtained from dogs with this disease. Analysis of these samples is nearing completion and further studies will be done during experimental transmission of the illness.

Viral Hepatitis. Gray and Barron^{11/} in 1943, described abnormal serum protein electrophoretic patterns in patients with acute viral hepatitis. Ricketts and Sterling^{12/} in 1949 not only studied patients during the acute stage of the disease but performed late follow-up studies and found that 8 of 11 patients who had clinically recovered from acute viral hepatitis had abnormalities of their serum protein electrophoretic patterns 10-36 months after the acute episode. Five of the eight patients who were noted to have abnormal patterns had elevated β -globulin fractions.

In our laboratory, the typical serum protein electrophoretic patterns of 12 patients with acute infectious hepatitis were characterized by an increase in γ -globulin and a decrease in the albumin fraction. The β -globulin fraction was at the upper limit of normal or increased in most cases, but the impressive characteristic finding was the β -globulin "spike" extending high above the α -globulin fractions in half of the patients. In most cases of acute infectious hepatitis the γ -glycoprotein was increased; the β -glycoprotein fraction was

normal or slightly increased; in no case was a β -glycoglobulin "spike" present. The total serum proteins were normal in most cases, as were the α -globulin and α -glycoglobulin fractions.

To investigate the efficacy of serum electrophoresis as a screening test in the diagnosis of previous hepatitis or hepatitis carriers, a double blind study was instituted in which serum proteins and glycoproteins were measured in Red Cross blood donors. The 300 samples studied consisted of sera from rejected blood donors (rejected because of a past history of jaundice, malaria, or hepatitis), acceptable blood donors, duplicates of rejected and acceptable blood donors, known normals, and known abnormals. Serum protein electrophoresis revealed a β -globulin "spike" in many of the sera in the presence of normality in the other serum protein fractions. Elevations of β -glycoglobulin were noted less often and were not as prominent as those seen in serum protein electrophoretic patterns. This study was recently completed. Preliminary analysis of the data revealed increased and/or spiked β -globulin and/or β -glycoglobulin which allowed the correct diagnosis of past hepatitis in about 50% of the blood donors with a past history of hepatitis. About 80% of the 15 patients with a past history of malaria were diagnosed as having past hepatitis; the other 20% were interpreted as being normal. At the present time we are analyzing this data more rigorously and will attempt to modify the criteria for diagnosis with the hope of increasing accuracy.

Hepatic Cirrhosis and Obstructive Liver Disease. Serum protein and glycoproteins have been measured in a few patients with hepatic cirrhosis and obstructive liver disease.

In the patients with hepatic cirrhosis, serum protein and glycoprotein patterns were characterized by decreases in serum albumin, and marked increases in γ -globulin and γ -glycoglobulin with "bridging" of the β - and γ -globulins and γ -glycoglobulins. The total serum protein as well as the α -proteins and α -glycoprotein fractions were normal to slightly decreased.

Serum protein and glycoprotein patterns in obstructive liver disease were characterized by increases in β -globulin and β -glycoglobulin fractions and marked increases in both α -globulin and α -glycoglobulin fractions with the α -glycoglobulin fractions being more prominent than the α -globulins. These changes are markedly different from those seen in infectious hepatitis and may be of diagnostic importance in differentiating hepatitis from congenital or acquired biliary obstruction.

Infectious Canine Hepatitis (ICH). Experimentally-induced ICH in dogs was characterized by marked increases in the α_2 -globulin, and α_2 -glycoglobulin fractions in < 72 hr. Other less prominent alterations of serum proteins and glycoproteins noted in α_1 , β , and albumin fractions are being evaluated at the present time.

In other studies. Alterations in serum proteins and glycoproteins have been observed in women taking antioviulatory drugs and in patients with streptococcal pharyngitis, infectious mononucleosis, influenza, and a variety

of other acute and chronic infectious and noninfectious diseases. Although these studies are incomplete, preliminary analysis of the data revealed that bacterial infections were associated with marked increases in the α -globulins and α -glycoglobulins whereas viral infections were associated with only slight elevations of these fractions. Most women taking antiovlatory drugs were noted to have slight to moderate increases α_2 - and β -globulins and α_1 - and/or α_2 -glycoglobulins. No significant alterations of other serum protein or glycoprotein fractions were noted.

Serum glycoproteins and lipoproteins will be measured in 20 volunteers before and after the administration of a mixed vaccine (VEE-WEE-EEE) (Medical Division Project FY 69-8). In this study, the lipoprotein method described above will be compared with 2 other methods of lipoprotein analysis recently developed in other laboratories.

Summary, Part II:

Studies to investigate the nature of infection-induced serum glycoprotein changes in the human and animal host have been expanded to include the study of infection-induced changes in serum protein and lipoprotein electrophoretic patterns.

A rapid quantitative electrophoretic method using cellulose acetate strips for measuring serum and plasma lipoproteins has been developed. Since the serum protein and glycoprotein methods used in this laboratory also utilize cellulose acetate strips, the development of this method now allows a simultaneous comparison of serum protein, glycoprotein, and lipoprotein electrophoretic patterns from a single sample of serum.

A summary of studies performed to investigate infection-induced serum protein, glycoprotein, and lipoprotein changes in the human and animal host is as follows:

1. Demonstration of only slight glycoprotein changes in a group of volunteers vaccinated with VEE Vaccine who became moderately ill.
2. Demonstration of marked serum protein and glycoprotein alterations in dogs with Idiopathic Hemorrhagic Syndrome of unknown etiology.
3. Demonstration of increases in γ - and β -globulins and a decrease in albumin in patients with acute viral hepatitis. Glycoprotein alterations, although present, were less prominent.
4. Demonstration in a double blind study of an increase and/or "spike" in serum β -globulin and/or β -glycoglobulin which allowed a correct diagnosis of previous hepatitis in about 50% of blood donors with a past history of hepatitis.
5. Demonstration of a decrease in serum albumin, and a marked increase in γ -globulin and γ -glycoglobulin with "bridging" of the β - and γ -serum globulins and glycoglobulins in patients with hepatic cirrhosis.

6. Demonstration of serum protein and glycoprotein patterns in obstructive liver disease characterized by an increase in β -globulin and β -glycoglobulin fractions and a marked increase in both α -globulin and α -glycoglobulin fractions with the α -glycoglobulin fractions being more prominent than the α -globulins.

7. Demonstration of a marked increase in the α_2 -globulin and α_2 -glycoglobulins in < 72 hr in dogs with experimentally-induced infectious canine hepatitis.

8. Demonstration of alterations in serum proteins and glycoproteins in women taking antioviulatory drugs and in a variety of infectious and noninfectious diseases.

Publications:

1. Feigin, R. D., A. S. Klainer, and W. R. Beisel. 1968. Factors affecting circadian periodicity of blood amino acids in man. *Metabolism* 17:764-775.

2. Klainer, A. S., W. R. Beisel, and W. K. Atkins. Determination of serum glycoproteins on cleared cellulose acetate strips. *Amer. J. Clin. Path.* 50:137-141, 1968.

3. Klainer, A. S., D. F. Clyde, P. J. Bartelloni, and W. R. Beisel. 1968. Serum glycoproteins in experimentally-induced malaria in man. *J. Lab. Clin. Med.* 72:794-802.

4. Feigin, R. D., H. G. Dangerfield, and W. R. Beisel. 1969. Circadian periodicity of blood amino-acids in normal and adrenalectomized mice. *Nature* 221:94-95.

5. Klainer, A. S., P. F. Gilliland, W. J. Cirksena, P. J. Bartelloni, W. R. Beisel. 1969. Serum glycoproteins in naturally acquired malaria in man. *Arch. Int. Med.* (In press).

LITERATURE CITED

1. U. S. Army Medical Unit. 1 July 1967. Annual Progress Report, FY 1967, p. 39 to 48. Fort Detrick, Maryland.

2. U. S. Army Medical Unit. 1 July 1968. Annual Progress Report, FY 1968, p. 39 to 44. Fort Detrick, Maryland.

3. Feigin, R. D., R. F. Jaeger, R. W. McKinney, and Alevizatos, A. C. 1967. Live attenuated Venezuelan Equine Encephalomyelitis virus vaccine. II. Whole blood amino acid and fluorescent-antibody studies following immunization. *Amer. J. Trop. Med.* 16:769-777.

4. U. S. Army Medical Unit. 1 July 1968. Annual Progress Report, FY 1968, p. 105 to 114. Fort Detrick, Maryland.

5. Weisberger, A. S. 1967. Inhibition of protein synthesis by chloramphenicol. *Ann. Rev. Med.* 18:483-494.
6. Klainer, A. S., W. R. Beisel, and W. K. Atkins. 1968. Determination of serum glycoproteins on cleared cellulose acetate strips. *Amer. J. Clin. Path.* 50:137-141.
7. U. S. Army Medical Unit. 1 July 1968. Annual Progress Report, FY 1968, p. 1 to 10. Fort Detrick, Maryland.
8. Klainer, A. S., D. F. Clyde, P. J. Bartelloni, and W. R. Beisel. 1968. Serum glycoproteins and experimentally-induced malaria in man. *J. Lab. Clin. Med.* 72:794-802.
9. Klainer, A. S., P. F. Gilliland, W. J. Cirksena, P. J. Bartelloni, and W. R. Beisel. 1969. Serum glycoproteins in naturally acquired malaria in man. *Arch. Int. Med.* (In press).
10. Kohn, J. A. 1961. Lipoprotein staining method for zone electrophoresis. *Nature* 180:312-313.
11. Gray, S. J., and E. S. G. Barron. 1943. The electrophoretic analyses of the serum proteins in diseases of the liver. *J. Clin. Invest.* 22:191-200.
12. Ricketts, W. E. and K. Sterling. 1949. Electrophoretic studies of the serum proteins in virus hepatitis. *J. Clin. Invest.* 28:1477-1486.

ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)
Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents
Work Unit No. 096 01 010: Effect of Irradiation on Infection and Immunity
Reporting Installation: U.S. Army Medical Research Institute of Infectious
Diseases
Fort Detrick, Maryland
Divisions: Medical and Bacteriology
Period Covered by Report: 1 July 1968 to 30 June 1969
Professional Authors: Charles P. Craig, Major, MC
Reports Control Symbol: RCS-MEDDH-288(R1)
Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OL0883	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DISSEM INSTR ^a	9. SPECIFIC DATA- CONTRACTOR ACCESS	10. LEVEL OF SUM
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES: ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62706A		1B662706A096		01	
b. Contributory		62124011		1B622401A096		01	
c. Contributory		CDOG 1212b(9)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) Effect of irradiation on infection and immunity							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
003500 Clinical Medicine; 004900 Defense; 003200 BW, CW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
67 07		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: ^a NA				69		1	
c. TYPE:				FISCAL YEAR		10	
d. AMOUNT:				CURRENT		10	
e. KIND OF AWARD:				70		1	
f. CUM. AMT.							
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a USA Medical Research Institute of Infectious Diseases				NAME: ^a Medical & Bacteriology Divisions USA Medical Research Institute of Infectious Diseases			
ADDRESS: ^a Fort Detrick, Md 21701				ADDRESS: ^a Fort Detrick, Md 21701			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Crozier, D.				NAME: ^a Pyfer, C. M.			
TELEPHONE: 301 663-4111 Ext 5233				TELEPHONE: 301 663-4111 Ext 4158			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered.				ASSOCIATE INVESTIGATORS			
				NAME: Craig, C. P.			
				NAME:			
22. KEY WORDS (Precede EACH with Security Classification Code)							
(U) Irradiation; (U) Immunity; (U) Infectious diseases; (U) Encephalitis, equine (VEE); (U) Yellow fever							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Investigate interrelationships between acute or chronic irradiation and disease processes.							
24. (U) Acute or protracted whole body irradiation is delivered to selected animal species before, simultaneously with, or after infection. Clinical and immune responses are observed and measured serially.							
25. (U) 68 07 - 69 06 - Mice were irradiated with 400 R 24 hr before immunization with varying doses of 2 vaccine viruses, VEE vaccine strain and yellow fever, 17-D. Seven and 14 days later they were challenged with virulent viruses, Trinidad VEE and Asibi yellow fever respectively. It is suggested that irradiation may suppress immunity by interfering with VEE virus replication. There is a possibility that increased dosage greater than 1,000 median infectious doses might override the depression in immune responses observed.							
Publications: Radiat. Res. 35:451-457, 1968; 36:98-106, 1968. J. Reticuloendothel. Soc. 5:582, 1968.							

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 010: Effect of Irradiation on Infection and Immunity

Description:

Earlier studies^{1,2/} documented a delay in appearance of hemagglutination inhibiting (HI) and neutralizing antibodies to Venezuelan equine encephalitis (VEE) in nearly lethally irradiated monkeys immunized with attenuated VEE vaccine. Comparable effects were not found under similar conditions using yellow fever vaccine strain (17-D). Irradiation did not affect the response to 17-D.

Because the routine immunizing dose of VEE vaccine contained 10^3 median mouse intraperitoneal immunizing doses (MIPID₅₀) of virus, and 17-D contained 10^5 MIPID₅₀, the effect of varying dosage of vaccine on immune responses of normal and irradiated (400 R, 24 hr before) mice was studied.

Mice were given 10^1 thru 10^3 MIPID₅₀ VEE vaccine or 10^1 thru 10^5 MIPID₅₀ 17-D vaccine subcutaneously. All animals were challenged IP with 10^3 LD₅₀ Trinidad strain VEE or intracerebrally (IC) with Asibi strain yellow fever virus, respectively, 7 or 14 days after immunization. Radiation had an apparent effect in VEE-vaccinated mice at both 7 and 14 days, and in normal mice at 7 days (Table I). No protection was induced in any of the yellow fever immunized; however, only 50% of control mice were protected at the highest vaccine dose used (Table II). It must be noted that the 17D vaccine used was of low potency.

TABLE I. EFFECT IN MICE OF 400R IRRADIATION 24 HR BEFORE IMMUNIZATION WITH VEE VACCINE ON SURVIVAL AFTER IP CHALLENGE WITH TRINIDAD VEE VIRUS.

VACCINE DOSE MIPID ₅₀	NO. IN EACH GROUP	SURVIVORS POSTCHALLENGE			
		Day 7		Day 14	
		400R	No R	400R	No R
10	12	0	6	3	10
100	12	1	10	12	10
1000	12	6	9	9	12

TABLE II. EFFECT IN MICE OF 400R IRRADIATION 24 HR BEFORE IMMUNIZATION WITH 17-D YELLOW FEVER VACCINE ON SURVIVAL AFTER IC CHALLENGE WITH ASIBI YELLOW FEVER VIRUS

VACCINE DOSE MIPID ₅₀	NO. IN EACH GROUP	SURVIVORS POSTCHALLENGE					
		Day 7			Day 14		
		400R	No	R	400R	No	R
10	24	0	0		0	0	
100	24	0	1		0	0	
1000	36	0	1		0	0	
10000	12	0	1		0	1	
100000	12	0	6		1	6	

These studies suggest that by increasing dosage of live VEE vaccines, irradiation depression of immune responsiveness may be overridden. In light of the known rapid proliferation of this virus vaccine *in vivo*, these differences suggest that the irradiation depression of immunity may be partially related to irradiation induced interference with virus replication as well as the well known cytotoxic effect of x-ray on the reticuloendothelial system.

It is also apparent that the time courses of immune response to VEE vaccine and 17-D in mice are vastly different.

Summary:

In mice it is suggested that 400R irradiation may suppress immunity by interfering with VEE virus replication. It is possible that by increasing the dosage of live VEE vaccine the irradiation depression of immune response may be overridden.

Publications:

1. Reynolds, S. L., C. P. Craig, H. W. Whitford, J. Airhart, and E. V. Staab. 1968. Antibody responses in rhesus monkeys exposed to whole body x-irradiation. *Radiat. Res.* 32:451-457.
2. Reynolds, S. L., H. W. Whitford, N. R. Blemly, E. V. Staab, and C. P. Craig. 1968. Effects of x-irradiation on the immune response of guinea pigs to Q fever vaccine. *Radiat. Res.* 36:98-106.
3. Staab, E. V., and C. P. Craig. 1968. Reticuloendothelial system function in guinea pigs infected with attenuated Venezuelan equine encephalitis virus. *J. Reticuloendothel. Soc.* 5:582

LITERATURE CITED

1. U. S. Army Medical Unit. 1 July 1967. Annual progress report, FY 1967, p. 151 to 159. Fort Detrick, Maryland.
2. U. S. Army Medical Unit. 1 July 1968. Annual progress report, FY 1968, p. 45 to 49. Fort Detrick, Maryland.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 011: Biophysical Studies of Pathogenic Microorganisms

Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland

Division: Physical Sciences

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Author: Anne Buzzell, Ph.D.

Reports Control Symbol: RCS-MEDDH-288 (R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
				DA OLO810	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8A. DISB'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES:*		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62706A		1B662706A096		01	
b. CONTRIBUTING		62124011		1B622401A096		01	
c. CONTRIBUTING		CDOG 1212b(9)					
11. TITLE (Precede with Security Classification Code)*							
(U) Biophysical studies of pathogenic microorganisms							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS*							
003500 Clinical Medicine; 004900 Defense; 003200 BW, CW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
62 02		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (In thousands)	
b. NUMBER:*				FISCAL YEAR		2	
c. TYPE:				CURRENT		30	
d. AMOUNT:				70		2	
e. KIND OF AWARD:				f. CUM. AMT.		30	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME:* USA Medical Research Institute of Infectious Diseases				NAME:* Physical Sciences Division			
ADDRESS:* Fort Detrick, Md 21701				ADDRESS:* USA Medical Research Institute of Infectious Diseases			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Crozier, D.				NAME:* Buzzell, A.			
TELEPHONE: 301 663-4111 Ext 5233				TELEPHONE: 301 663-4111 Ext 6237			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered.				ASSOCIATE INVESTIGATORS			
				NAME: Quin, S.			
				NAME:			
22. KEYWORDS (Precede Each with Security Classification Code)							
(U) Biophysics; (U) Encephalitis, equine (VEE); (U) Pneumococcus; (U) Microscopy, electron; (U) Liver; (U) Viruses; (U) Coliphages; (U) Staining (negative); (U) Thymus							
23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Characterize pathogenic microorganisms in terms of physical properties, host cell interaction, and similarity between strains.							
24. (U) Methods are under development to concentrate virus particles rapidly from biologic fluids for electron microscopic study and to use the electron microscope for studies of very early changes in host cells during infection.							
25. (U) 68 07 - 69 06 - Transfer of virus with staining from Sepraphore III, had become erratic. Careful analysis of the interaction of stain with the Sepraphore III material, based on ideas from a literature survey, has revealed a number of sources of trouble. A new procedure has recently been evolved which has circumvented the difficulties yielding reproducible transfer with clearly stained images. The procedure should be applicable to use with millipore filters so that new procedures for concentrating the virus can be tried.							
A study of host response to infection by electron microscopy of the pneumococcal infected rat has revealed interesting early changes in the thymus.							

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 011: Biophysical Studies of Pathogenic Microorganisms

Description:

To characterize infectious microorganisms in terms of biophysical properties and host-cell interactions.

Progress, Part I:

Advances have been made in developing a rapid electron microscopic assay for virus particles in unpurified dilute suspensions. The assay is derived from that of Sharp^{1/}. As described in earlier reports^{2,3/}, a much simpler procedure for transferring the virus from agar to the electron microscope grid has been evolved which incorporates negative staining, thus facilitating identification of the virus. Furthermore the new procedure is not limited to transfer from agar. Since virus can be deposited on materials such as millipore filters new concentration procedures can be tried which may greatly increase the sensitivity of the assay from the present limit of about 10^6 particles/ml.

As reported last year,^{2/} the low flow rate of fluid through millipore filters necessitated use of high concentrations of the negative stain, sodium phosphotungstate, and results became erratic apparently because of aggregation of the salt.

Subsequent work has been confined to Sepraphore III, similar to millipores chemically but through which fluid flows readily. Despite ready passage of water, transfer of virus and negative stain continued to be erratic. Gradually through systematic testing of ideas obtained from a literature survey the nature of the complex interactions of the stain (and other related stains) and Sepraphore III have been unravelled. A procedure now being tested has given encouraging results, so far reproducible, in which the negative stained images approach the clarity obtained when virus was being transferred from agar. It is hoped, and seems likely, that the procedure can be adapted for use with millipore filters so that work on new virus concentration procedures can be started.

Summary, Part I:

A procedure which appears reliable has been evolved using Sepraphore III as the rigid depository material in the concentration

step of the electron microscopic assay for virus particles. The negative-stained images obtained of virus washed from sephaphore with phosphotungstate solution approach in clarity the images of virus washed from agar. It is expected that the new procedure will be adaptable to use with millipore filters.

Progress, Part II:

Host response to infection, with pneumococcus infected rats as a model, is being studied by electron microscopic investigation of ultrastructural changes in various organs. Initial study of the thymus showed striking changes which occurred within 3 hr after inoculation with 10^6 organisms. Parallel investigation of the liver is planned as time permits. The program initiated with Dr. W. R. Beisel is expected to be continued in collaboration with COL J. F. Metzger of the Pathology Division.

Summary, Part II:

In the electron microscopic study of pneumococcus-infected mice early changes observed in the thymus appear quite interesting. It is planned to continue the study in collaboration with the Pathology Division and broaden the investigation to other organs.

Publications:

None

LITERATURE CITED

1. Sharp, D. C. 1949. Enumeration of virus particles by electron microscopy. Proc. Soc. Exp. Biol. Med. 70: 54-59.
2. U. S. Army Medical Unit. 1 July 1967. Annual Progress Report, FY 1967, p. 67 to 71. Fort Detrick, Maryland.
3. U. S. Army Medical Unit. 1 July 1968. Annual Progress Report, FY 1968, p. 51 to 55. Fort Detrick, Maryland

ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 012: Biophysical Studies of Bacterial Toxins and
Other Inert Molecules

Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland

Division: Physical Sciences

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Author: Anne Buzzell, Ph.D.

Reports Control Symbol: RCS-MEDDH-288 (R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
				DA OLO889	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8. DISB'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
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a. PRIMARY	62706A	1B662706A096		01	012		
b. CONFIDENTIAL	62124011	1B622401A096		01			
c. CONFIDENTIAL	CDOG 1212b(9)						
11. TITLE (Precede with Security Classification Code)*							
(U) Biophysical studies of bacterial toxins and other inert molecules							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS*							
003500 Clinical Medicine; 004900 Defense; 003200 BW, CW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
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17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING			
b. NUMBER: NA				69		1	
c. TYPE:				FISCAL YEAR		b. FUNDS (in thousands)	
d. AMOUNT:				CURRENT		10	
e. KIND OF AWARD:				70		1	
f. CUM. AMT.						10	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: USA Medical Research Institute of Infectious Diseases				NAME: Physical Sciences Division			
ADDRESS: Fort Detrick, Md 21701				ADDRESS: USA Medical Research Institute of Infectious Diseases			
				Fort Detrick, Md 21701			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Crozier, D.				NAME: Buzzell, A.			
TELEPHONE: 301 663-4111 Ext 5233				TELEPHONE: 301 663-4111 Ext 6237			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Toxin; (U) Metabolism; (U) Biophysics; (U) Ultracentrifugation; (U) Latex particles							
23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Characterize various toxins or other inert molecules which have biologic activity.							
24. (U) Various purified and partially purified elements of toxins will be examined by chromatography, ultracentrifugation, electron microscopy, etc.							
25. (U) 68 07 - 69 06 - A simple method of measuring partial specific volumes of proteins isolated by density gradient centrifugation at low concentrations in the presence of sucrose has been applied to sedimentation of large particles having a density close to that of the sucrose. A fundamental improvement has been achieved in the method for obviating the need for precise temperature control. Density estimations accurate to the sixth decimal place can now be made. The increased sensitivity has revealed density variation in the particles arising from metal ion binding. This chelation property of the particles is being studied to see how it affects the ability of the particles to bind protein.							

*Available to contractors upon originator's approval.

DD FORM 1400-1 (FOR ARMY USE)

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 012: Biophysical Studies of Bacterial Toxins and Other Inert Molecules

Description:

To characterize various toxins or other inert molecules which have biological activity.

Progress:

During the biophysical study of components of anthrax toxin^{1/} resolved by sucrose density gradient centrifugation, the need became apparent for a new method of measuring partial specific volumes of proteins in the presence of high concentrations of sucrose, since the toxin components could not be readily freed from sucrose. In a previous report^{2/} a simple method was described that involves measuring the sedimentation rate of large spherical latex particles in a sucrose solution adjusted to have a density close to that of the particles. In the experiments described previously the poorer accuracy of 1×10^{-5} gm/ml achieved was ascribed to the difficulty of maintaining adequate temperature control (without expensive modifications in the Spinco Model E ultracentrifuge). Since then a procedure has been devised which eliminates the need for precise temperature control and variations of several degrees have no detectable effect on the calculated density increments. With temperature variations in density eliminated, a new source of variation became apparent which proved to arise from variation in numbers of metal ions attached to the particles, apparently by chelation. Details of the metal binding are currently under study so that maximum uniformity and reproducibility in particle density can be achieved.

In the previous report,^{2/} it was pointed out that certain immunological procedures of interest to the unit used similar latex particles coated with antibody or antigen. Preliminary estimates of protein (bovine serum albumin) binding indicated that saturation of the particles occurred in 0.003% protein solution and produced a particle density increment of 1×10^{-4} gm/ml. In view of the new findings concerning chelation, further study of protein binding is clearly in order. It seems highly likely that binding will depend on what, and how many, metal ions are attached to the particles and systematic study should yield information valuable for predicting conditions of maximum protein binding for immunological purposes, and conditions of minimum binding for partial specific volume studies.

Summary:

The technique for determining partial specific volumes of protein by measuring the sedimentation rate of large latex particles in solutions of the protein has been improved by eliminating the need for close temperature control. With the resulting increase in sensitivity, some interesting properties of the latex particles have been revealed which probably relate to the variable tendency of particles to bind protein.

Publications:

None

LITERATURE CITED

1. Buzzell, A. 1967. Structural model for the lethal components of anthrax toxin based on ultracentrifuge studies. Fed. Proc. 26:1522-1526.
2. U. S. Army Medical Unit. 1 July 1968. Annual Progress Report FY 1968. p.57 to 60. Fort Detrick, Maryland.

ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 401: Effect of Bacterial and Viral Infections on Host
Cell Biosynthetic Mechanisms

Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland

Division: Physical Sciences

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Authors: Michael C. Powanda, Captain, MSC (I, II, III)
Robert S. Pekarek, Ph.D. (IV)
Robert P. Nalewaik, Lieutenant, USNR (V)
William L. Steinhart, Captain, MSC (VI)

Reports Control Symbol: RCS-MEDDH-288 (R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
				DA OL0809	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8. DISSEM INSTR*	9. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUMMARY
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES*		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
						WORK UNIT NUMBER	
a. PRIMARY		62706A		1B662706A096		01	
b. CONTRIBUTING		62124011		1B622401A096		01	
c. CONTRIBUTING		CDOG 1212b(9)					
11. TITLE (Precede with Security Classification Code)*							
(U) Effect of bacterial and viral infections on host cell biosynthetic mechanisms							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS*							
003500 Clinical Medicine; 004900 Defense; 003200 BW, CW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
62 10		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER:*				FISCAL YEAR			
c. TYPE:				CURRENT			
d. AMOUNT:				70		40	
e. CUM. AMT.				4		40	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME:*				NAME:*			
USA Medical Research Institute of				Physical Sciences Division			
Infectious Diseases				USA Medical Research Institute of			
ADDRESS:*				ADDRESS:*			
Fort Detrick, Md 21701				Infectious Diseases			
				Fort Detrick, Md 21701			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME:				NAME:*			
Crozier, D.				Powanda, M. C.			
TELEPHONE:				TELEPHONE:			
301 663-4111 Ext 5233				301 663-4111 Ext 5214			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered.							
22. KEYWORDS (Precede EACH with Security Classification Code)				ASSOCIATE INVESTIGATORS			
(U) Nucleotides; (U) Diurnal rhythm; (U) Penumococcus; (U) Tryptophan; (U) Salmonella;				NAME:			
(U) RNA; (U) Tularemia; (U) Mycoplasma; (U) Chromatin template activity				Pekarek, R. S.			
				NAME:			
				Steinhart, W. L.			
				DA			
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To study alterations in nucleotide metabolism, RNA biosynthesis, and template activity of isolated chromatin in host cells during infection.							
24. (U) Total nicotine-adenine dinucleotide (NAD) is measured in the tissues of mice subjected to a variety of conditions including infection with pneumococcus. The incorporation of radioactively labeled precursors of RNA into RNA isolated from various subcellular fractions is measured in the host during infection.							
25. (U) 68 07 - 69 06 - A diurnal variation in total NAD content has been observed in mouse liver and spleen. The changes in liver NAD and NADH content of mice infected with pneumococci appear to be the result of decreased food intake. Changes in liver NAD do not appear to be responsible for the protective effect of supplemental tryptophan against <u>Salmonella typhimurium</u> infection in mice. Marked decreases in the biosynthesis of RNA have been observed in the tissues of mice infected with <u>Pasteurella tularensis</u> . In <u>Mycoplasma</u> the incorporation of precursors suggests the presence of rapidly turning over "DNA-like" RNA, indicating that the biosynthesis of RNA in these cells is similar to that of bacteria and mammalian cells. Techniques are presently being developed for studying the template activity of chromatin from tissues of mice during pneumococcal infection.							
Publication: Biochim. Biophys. Acta 174:761-763, 1969.							

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 401: Effect of Bacterial and Viral Infections
on Host Cell Biosynthetic Mechanisms

Description:

To study alterations in nucleotide metabolism, RNA biosynthesis, and template activity of isolated chromatin in host cells during infection.

Progress, Part I:

A successful host response to the stimulus of infection appears to require the expenditure of energy. Nicotinamide-adenine-dinucleotides, (NAD); its reduced form, (NADH); and nicotinamide-adenine-dinucleotide phosphate, (NADP); its reduced form (NADPH) are essential to both the generation of energy (glycolysis, electron transport) and its use (e.g., lipid and steroid synthesis).

Studies on normal, male, CD-1 mice (Charles River) on a 0630-1830 hours lights on, 1830-0630 lights off schedule revealed a statistically significant diurnal variation in total hepatic NAD (M/m tissue), with high values occurring between 2000 and 0400 hours and low values between 0800 and 1600 hours. This agrees with previous work from this unit which gave evidence that tryptophan oxygenase, an enzyme whose action can yield precursors to the pyridine nucleotides, exhibits a similar diurnal variation.^{1/} A similar pattern of variation in total NAD, though of smaller magnitude, has been observed in spleen; the kidney seems to exhibit oscillations in levels.

Summary, Part I:

A daily variation exists in the total NAD content of liver and spleen tissue of normal male mice on a 12-hr on, 12-hr off light schedule. This pattern may not hold true for all tissues since under the same conditions the kidney shows multiple slight oscillations rather than a distinct peak and trough.

Progress, Part II:

Preliminary investigation into the effects of massive pneumococcal infection (approximately 10^6 organisms per animal, causing death <24 hr) on mouse hepatic pyridine nucleotide metabolism indicates that in the first 12 hr postinfection the decrease seen in total NAD (from 0.587 ± 0.034 to 0.459 ± 0.032 M/m tissue) can be accounted for by the lessened intake of food resulting from infection. This is evidenced by the observation that fasted controls show similar changes in NAD content over the same period (from 0.628 ± 0.017 to 0.441 ± 0.022).

In the first 12 hr after infection total hepatic NADH increases in both the infected animals (from 0.260 ± 0.024 to 0.365 ± 0.003 M/gm) and the fasted controls (from 0.241 ± 0.007 to 0.316 ± 0.025). However, the difference between the infected and control animals in this study was not always statistically significant.

Summary, Part II:

The early changes in total NAD and NADH seen in mice infected with a massive dose of pneumococci appear for the most part, to be the result of decreased food intake due to illness.

Progress, Part III:

Experiments are underway to investigate variations in tryptophan metabolism in mice in response to Salmonella typhimurium infection. Recent evidence indicates that diets supplemented to the 3% level with respect to tryptophan instead of the normal 0.22% give significant protection against S. typhimurium in mice.^{2/}

The protection afforded by enhanced dietary tryptophan may be (1) mediated via the resulting increase in liver tryptophan oxygenase activity and NAD, (2) the result of some other substance formed from tryptophan, or (3) a property of the tryptophan molecule itself.^{3/}

To test the first possibility, pharmacologic amounts of nicotinamide have been given to mice in their water supply so as to alter the pyridine nucleotide content and tryptophan oxygenase activity of the liver without increasing the dietary intake of tryptophan. The levels of nicotinamide used resulted in smaller increments in enzyme activity but approximately the same level of NAD as did the 3% tryptophan diet. In the one study completed to date, nicotinamide failed to enhance survival.

Summary, Part III:

Preliminary evidence suggests that the protective effect of supplemental dietary tryptophan against S. typhimurium in mice does not seem to operate via increases in hepatic NAD content.

Progress, Part IV:

The effect of an intracellular bacterial infection on RNA biosynthesis in the host was studied. Charles River CD-1 male mice, weighing 25 - 30 gm, were infected with the attenuated vaccine strain of Pasteurella tularensis.¹⁴ Two hours prior to sacrifice 5 μ C of RNA precursor, orotic acid-¹⁴C, was injected intraperitoneally. At 24 and 48 hr the animals were sacrificed, and nuclear, microsomal, and soluble RNA fractions were isolated from the kidney, liver, and spleen for analysis. Specific activities, expressed as cpm/OD, were determined for each fraction isolated in both infected and control animals.

At 24 hr marked decreases in specific activities of both nuclear and cytoplasmic RNA fractions in all 3 organs were observed when compared to control values. Decreases, in the order of 54 and 80% of control values, were observed in the nuclear fraction of the liver and kidney respectively. At 48 hr cytoplasmic fractions were of the same approximate magnitude as controls, and the specific activities of the nuclear fractions were found to be slightly increased above controls.

Summary, Part IV:

The biosynthesis of nuclear and cytoplasmic RNA was studied in the animal host infected with P. tularensis. Marked decreases in specific activities of nuclear RNA were observed in liver, kidney, and spleen 24 hr postinfection. At 48 hr nuclear RNA was found to be only slightly increased above control values.

Progress, Part V:

Although pleuropneumonia-like organisms (Mycoplasma; PPL0) are able to replicate in cell-free media, their small genome size and complex nutritional requirements have raised questions regarding their biosynthetic capabilities. Previous work^{4/} has suggested that protein biosynthesis in PPL0 follows the same basic mechanism as that found in bacterial, fungi, plants and animals. These studies indicated the presence of DNA-dependent RNA synthesis. This work has raised questions concerning RNA formation in Mycoplasma. Non-pulse and pulse-labeled experiments were designed in an attempt to answer these questions.

In non-pulsed-labeled experiments, uridine-2-¹⁴C was rapidly incorporated in a linear fashion in whole cells and in trichloroacetic acid-(TCA)-hydrolyzed RNA of Mycoplasma laidlawii B. Similar incorporation of labeled uridine was observed in phenol extracted RNA.

Pulse-labeled experiments were performed in two different ways. When PPLO were grown, harvested, washed in medium, resuspended in fresh medium, and pulse-labeled for 15 min over a 4-hr period, the kinetics of RNA synthesis could be demonstrated. A rapid rise in the specific activity (cpm/OD 260 mμ) of RNA was seen within the first 60 min. Thereafter, there was a "leveling off" of specific activity which approached an equilibrium state by 90 min. This equilibrium state persisted for the duration of the experimental period. The initial rapid rise suggested a response to a change in environment, whereas the equilibrium state represented the rate characteristic of RNA synthesis in the fresh medium.

When samples were drawn periodically from an actively growing PPLO culture and pulse-labeled for 15 min, RNA synthesis was found to remain constant with regard to specific activity. Here, there was no environmental change, so a definitive rate had already been established and was maintained.

Calculation of the μmoles of labeled uridine indicated a continuous step-wise increase over the time periods studied, suggesting continuous incorporation of the precursor for RNA formation.

Addition of the inhibitor Actinomycin D with the pulse-label resulted in an immediate cessation of RNA production.

The rapid incorporation of labeled uridine and the action of Actinomycin D raised questions as to which type of RNA takes up most of the label in the above studies. In an attempt to answer these questions, methylated bovine serum albumin (MSA) columns were utilized. NaCl phosphate buffered solutions were used in a linear gradient over a 0.1M to 1.0M range. Using such a system, total RNA extracted by the phenol procedure could be fractionated into its 4, 16 and 22S components, as determined by spectrophotometry at 260 mμ. The gradient range was changed to 0.2M to 1.0M to differentiate better the 16 and 22S peaks. Using a windowless gas-flow isotope counter, labeled uridine was present in 4 peaks. These peaks were in the area of the 16 and 22S peaks which are ribosomal RNA. Other investigators,^{5/} using other organisms, have demonstrated similar results. Their results and those found here suggest that the isotope peaks are "DNA-like" RNA ("messenger" RNA). The increase in label over 10-min periods indicates a half-life of 2 min for this RNA which corresponds to a value calculated by another investigator.^{6/} Actinomycin D did not decrease ultraviolet readings appreciably, but did produce a significant drop in the incorporation of the uridine label.

L-929 mouse fibroblast cells, grown in suspension, have been fractionated to obtain cytoplasmic, microsomal, and nuclear RNA. These RNA's were run on MSA columns. Results are presently being evaluated. Studies of the uptake of labeled uridine by these cells and the effect of Actinomycin D are planned for the future. All this work is preliminary to a study of the interaction between the host (L-cell) and the agent (PPLO) for host-agent response studies.

Summary, Part V:

In Mycoplasma, the incorporation of labeled uridine and the effect of Actinomycin D suggest the presence of a rapidly turning over "DNA-like" RNA. It appears that the formation of RNA in these cells is similar to that of bacteria and mammalian cells.

Progress, Part VI:

Studies have been initiated to define the metabolic response of pneumococcus-infected mice by looking for changes in (1) the rate of synthesis of DNA-like RNA, and (2) the synthesis of qualitatively different RNA molecules, including their contribution to the total RNA pool. In vitro synthesis of RNA will be studied using the Micrococcus lysodeiketicus RNA polymerase and isolated mouse chromatin template from various tissues. The RNA products will be compared by hybridization-competition capacity for binding to deproteinized DNA of mouse embryos.

At present techniques are being developed to study these aspects. The conditions for the infection of mice and a method for isolation of mouse liver chromatin have already been defined.

Summary, Part VI:

Techniques are presently being developed for studying the template activity (gene expression) of chromatin isolated from various tissues of mice during infection by D. pneumoniae.

Publications:

1. Kehoe, J. M., G. Lust, and W. R. Beisel. 1969. Lymphoid tissue-corticosteroid interaction: An early effect on both Mg^{2+} - and Mn^{2+} -activated RNA polymerase activities. *Biochim. Biophys. Acta* 174:761-763.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 403: Host-parasite Relationships in Arbovirus Infections

Report Installation: U. S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

Division: Animal Assessment

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Authors: Ronald E. Vaughn, Captain, VC (I)
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Reports Control Symbol: RCS-MEDDH-288 (R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OL0812	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8A. DISSEM INSTR ^a	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
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10. NO./CODES: ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62706A		1B662706A096		01	
b. secondary		62124011		1B622401A096		01	
c. tertiary		CDOC 1212b(9)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) Host-parasite relationships in arbovirus infections							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
003500 Clinical Medicine; 004900 Defense; 003200 CW, BW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
61 07		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: ^a				FISCAL YEAR		c. FUNDS (in thousands)	
c. TYPE:				CURRENT YEAR		d. FUNDS (in thousands)	
e. KIND OF AWARD:				f. CUM. AMT.		e. FUNDS (in thousands)	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered.				ASSOCIATE INVESTIGATORS			
				NAME: Vaughn, R. E.			
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				DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Encephalitis (VEE, EEE); (U) Virus diseases; (U) Arboviruses; (U) Yellow fever; (U) Teratology							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Investigate various interactions between animal hosts and arboviruses.							
24. (U) Study transmissibility and susceptibility to arboviruses in a variety of animal species.							
25. (U) 68 07 - 69 06 - TC-83 strain of VEE was used to evaluate the effects of <u>in utero</u> viral transmission in the Swiss-Webster mouse. A decreased litter size, decreased number of live births, and a decreased survival until weanling age was seen in neonates born to dams infected on days 10-14 of gestation. Decreased survival to weanling age was also shown when dams were infected from days 16-19 of gestation. No effects on conception or gestation time were demonstrated. Serological responses were the only evidence of infection seen when a burro and a pony were inoculated with Asibi strain yellow fever virus by the intracerebral or intravenous routes.							

^a Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 403: Host-parasite Relationships in Arbovirus Infections

Description:

Investigate various interactions between animal hosts and arboviruses.

Progress, Part I:

The effect of in utero transmission of Venezuelan equine encephalitis (VEE) in mice has been studied. Previous studies have indicated that VEE could be transmitted to the fetus by intraperitoneal (IP) inoculation of the dam.^{1/} Data have been obtained on the effect of the virus on the products of conception. Initially, 113 pregnant mice were selected to determine the normal gestation time, average conception rate, number of mice born, number born alive, number weaned and the average birth and weaning weight of the Fort Detrick strain of Swiss-Webster mouse.

Pregnant mice were then inoculated with 1,000 median suckling mouse intracerebral lethal doses (SMICLD₅₀) of the TC-83 strain of VEE (TC-83) on days 0 - 19 of gestation. Neither an increase nor a decrease in gestation time was observed when TC-83-injected mice were compared to saline-injected control mice; nor was any significant difference seen in conception rate. However, a marked effect on litter size, live birth and neonatal survival was observed.

Mean values for approximately 30 litters per day are shown in Table I. The mean number born per litter begins to decrease on day 10 and continues to decline to a minimum on day 12, with a return to normal range on day 14. Thus, a significant drop in litter size is seen when pregnant mice are injected late in the 2nd trimester and early in the 3rd trimester of pregnancy. Since implantation of the fetus has already occurred, this then must represent fetal abortion and/or fetal resorption. The effect is not an all-or-none phenomenon, in that the percentage of dams giving birth remains the same and only an overall decrease in litter size occurred.

Column 2 of Table I represents the number born alive, divided by the total number born. The primary difference between this and the effect on litter size is that after the live birth ratio begins its decline on day 11, it

TABLE I. EFFECTS OF MATERNAL INJECTION WITH TC-83 VEE VIRUS ON FETUS AND NEONATE.

DAY OF PREGNANCY ON WHICH MICE WERE INJECTED	NO. BORN/ LITTER	NO. LIVE BIRTHS/LITTER	NO. OF NEONATES WEANED/ NO. BORN ALIVE
Controls	9.9	9.7	.867
1	9.3	9.1	.840
2	9.9	9.4	.845
3	9.5	9.3	.858
4	8.3	7.9	.931
5	9.4	9.2	.920
6	9.1	8.9	.775
7	8.8	8.5	.874
8	8.8	8.4	.815
9	8.9	8.6	.835
10	8.0	7.5	.573
11	6.1	4.8	.773
12	5.8	3.7	.760
13	6.5	4.6	.712
14	8.6	7.1	.635
15	8.7	7.5	.515
16	9.2	8.9	.184
17	10.0	10.0	.153
18	9.0	8.8	.447
19	10.3	10.2	.766

does not reach the normal range again until day 16.

Column 3 of Table I represents the ratio of the number of neonates weaned to the number born alive. Again starting on day 10, a significant decrease in this ratio is observed from days 10-18, with peak depressions occurring on days 10, 16 and 17. Thus, not only are litter sizes smaller and fewer pups born alive, but also fewer survive to weaning age when dams are infected on days 10-14 of gestation. Beginning with days 15 or 16, even though the normal number are born alive, a decreasing number of these survive to weaning age, thus suggesting that a different phenomenon may be involved in the effects observed on these different times of inoculation.

Since mice injected on days 15-18 had peak viremias on or around the day of parturition, this prompted the question of whether the young were being infected after birth by secretions of the dam, with resultant death early in life, or were they being infected in utero. To test this premise, newborn were removed from the infected dam at precisely the time of parturition and transferred to an uninfected control mouse, which likewise had just given birth. At the same time, the litter from the control mouse was transferred to the infected mouse and allowed to suckle. In this manner, the offspring of infected dams were exposed only to the secretions of an uninfected dam, and vice versa. Litters from infected dams were interchanged and litters from control dams were interchanged. In addition, dams which were not injected with TC-83 were allowed to keep their own offspring.

TABLE II. VIABILITY INDEX.

GROUP ^a /	INDEX BY DAY OF GESTATION ON WHICH MICE WERE INJECTED			
	15	16	17	18
I	0.95	0.92	0.88	0.97
II	0.35	0.18	0.41	0.68
III	0.44	0.22	0.44	0.69
IV	0.87	0.85	0.83	0.87

- a. I. Neonates from control dams placed with infected dams.
 II. Neonates from infected dams placed with control dams.
 III. Neonates from infected dams placed with other infected dams.
 IV. Neonates from control dams placed with other control dams.

In Table II, it may be seen that there is decreased viability in Groups II and III, which are the 2 groups involving offspring of dams which were infected with TC-83. On the other hand, no decrease in viability is seen in Groups I and IV, which are the 2 groups involving offspring from dams which were not infected with TC-83. This indicates that the cause of decreased viability of offspring from mothers injected with TC-83 on days 15, 16 and 17 is transmitted in utero, rather than by postnatal exposure to any secretions of the dams.

In order to examine both the degree of immunity and how it is acquired, all of the surviving weanlings from the experiment just described were challenged with 1,000 median mouse intraperitoneal lethal doses (MIPLD₅₀) of the Trinidad strain of VEE (Table III).

TABLE III. IMMUNITY OF SURVIVING WEANLINGS.

GROUP ^{a/}	NUMBER SURVIVED/NUMBER CHALLENGED		
	Day of Gestation on Which Mice Were Injected		
	16	17	18
I	44/56	66/73	22/22
II	11/11	36/38	28/71
III	9/12	36/39	42/45
IV	0/76	0/76	0/81

a. See footnote Table II.

Since all weanlings from Group IV, which consisted of control weanlings placed with control dams, died and the other 3 groups evidenced protection to a highly significant degree, we can say that mice receive protection both through placental experience and through colostrum. Because the time from injection of the dam to birth is too short for detectable circulating antibody in the dam, it is postulated that the protection observed in Group II may be other than specific antibody acquired from the dam.

Summary, Part I:

TC-83 strain of VEE was used to evaluate the effects of in utero virus transmission in the Swiss-Webster mouse. A decreased litter size, decreased number of pups born alive and a decreased survival until weanling age was seen in neonates born to dams infected on days 10-14 of gestation. Decreased survival to weaning age was also shown when dams were infected on days 16-19

of gestation. No effect on conception or gestation time was demonstrated.

Progress, Part II:

A burro and a pony were challenged IV and intracerebrally (IC), respectively, with virulent Asibi strain yellow fever virus. Neither animal responded clinically or hematologically, as determined by daily observation, temperature, complete blood count, serum glutamic oxalacetic transaminase, serum glutamic pyruvic transaminase, blood urea nitrogen, alkaline phosphatase, sodium, potassium and lactic dehydrogenase determinations.

Bleedings twice daily for 10 days revealed no viremia (no deaths at 10^{-1} injection into weanling mice). Response to the virus was evidenced by the neutralization of 1.8 logs of virus (at day 28) by the burro, and by the neutralization of 1.3 logs of virus (at day 21) by the pony.

Summary, Part II:

Serological responses were the only evidence of infection seen when a burro and a pony were inoculated with Asibi strain yellow fever virus by the IV or IC routes.

Publications:

None.

LITERATURE CITED

1. U. S. Army Medical Unit. 1 July 1968. Annual Progress Report, FY 1968. p. 67 to 73. Fort Detrick, Maryland.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 800: Biological Effects of Microbial Toxins

Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland

Divisions: Physical Sciences, Animal Assessment, &
Pathology

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Authors: David A. Rhoda, Captain, VC (I, II)
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Reports Control Symbol: RCS-MEDDH-288 (R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a DA OL0871	2. DATE OF SUMMARY ^a 69 07 01	REPORT CONTROL SYMBOL DD-R&E (AR) 636	
3. DATE PREV SUMRY 68 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY ^a U	6. WORK SECURITY ^a U	7. REGRADING ^a NA	8. DISSEM INSTR ^a DE	9. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES: ^a		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY		62706A	1B662706A096	01		800	
b. CONTRACTOR		62124011	1B622401A096	01			
c. CONTRACTOR		CDOG 1212b(9)					
11. TITLE (Precede with Security Classification Code) ^a (U) Biological effects of microbial toxins							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a 003500 Clinical Medicine; 004900 Defense; 003200 BW, CW, RW							
13. START DATE 66 10		14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:		EXPIRATION:		PRECEDING		b. FUNDS (2n thousands)	
b. NUMBER: ^a				69		30	
c. TYPE: NA		d. AMOUNT:		CURRENT		5	
e. KIND OF AWARD:		f. CUM. AMT.		70		30	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a USA Medical Research Institute of Infectious Diseases ADDRESS: ^a Fort Detrick, Md 21701				NAME: ^a Physical Sciences & Animal Assess Divs USA Medical Research Institute of Infectious Diseases Fort Detrick, Md 21701			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered.				ASSOCIATE INVESTIGATORS			
				NAME: Hobbs, C. H.			
				NAME: DA			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Enterotoxin; (U) Staphylococcus; (U) Adrenal hemorrhage (U) Vascular permeability							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To study the biological effects of microbial toxins.							
24. (U) The effects of SEB on vascular integrity is being evaluated by studying the production of adrenal hemorrhage in rabbits and by measuring the flux of radioiodinated serum albumin from plasma to lymph in monkeys. The fever dose for SEB by the aerosol route is being assessed in monkeys.							
25. (U) 68 07 - 69 06 - Attempts to produce adrenocortical hemorrhage by injection of SEB into male rabbits pretreated with ACTH have yielded inconstant results. SEB toxemia appears to cause a transient increase in vascular permeability in the monkey. Efforts to obtain the median fever dose of aerosol introduced SEB in monkeys have been unsuccessful to date because of erratic incidence of fever.							

^a Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 800: Biological Effects of Microbial Toxins

Description:

To study the biological effects of microbial toxins in the animal host.

Progress, Part I:

The production of adrenal hemorrhage in rabbits with 10 µg/kg intravenous (IV) of staphylococcal enterotoxin B (SEB) 4 hr after pretreatment with 20 units of ACTH was studied. Moderately severe hyperemia was demonstrated grossly and microscopically in 15 of 45 male rabbits weighing >3 kg. This observation was made as early as 15 min after injection of SEB. Since lesions seemed less frequent in female rabbits, most studies were conducted in males. Experimental results were inconclusive, since gross and microscopic evidence of hyperemia and hemorrhage was present in some control animals. Criteria for microscopic assessment of hemorrhages were: (1) occlusive sinusoidal thrombi; (2) focal necrosis of adrenal cortical cells; (3) focal aggregations of pseudo-eosinophils surrounding necrotic areas; (4) hemolysis of erythrocytes in thrombosed sinusoids; (5) coagulative necrosis in infarcted zones; and (6) nuclear fragmentation of pseudo-eosinophils in areas of thromboses. Six of 157 animals had foci of unequivocal adrenocortical necrosis; in 4, fragmentation of nuclei within these necrotic zones indicated that the lesion antedated the experimental injection of SEB. Based on the lack of clearcut experimental differences, additional work with this model system was discontinued.

Summary, Part I:

The production of adrenal hemorrhage in rabbits with IV SEB 4 hr after pretreatment with ACTH was studied. Due to a lack of differences no further work will be conducted.

Progress, Part II:

The effects of 300 µg/kg of SEB on vascular permeability is being studied in the rhesus monkey by measuring the transfer of radioiodinated human serum albumin (RISA) to thoracic duct lymph. A significant increase in the lymph: plasma ratio of RISA concentration occurred after SEB, reaching 0.9 by 3 hr and returning toward the normal value of about 0.5 by 10 hr. Lymph flow increased slightly, but did not appear to correlate with either the lymph:plasma ratio or the hypotension associated with the toxemia.

Summary, Part II:

SEB toxemia appears to cause a transient increase in vascular permeability in the rhesus monkey.

Progress, Part III:

To determine a median fever dose (FD₅₀) for SEB by the aerosol route, monkeys were exposed in a modified Henderson apparatus. To date, total dose used has been 0.1, 0.2, and 2.0 µg. Results are presented in a preliminary fashion in Table I. It is obvious that further work is required

TABLE I. OCCURRENCE OF FEVER FOLLOWING AEROSOL EXPOSURE OF MONKEYS TO VARYING DOSES OF SEB

TOTAL DOSE µg	FEVER ^a /	
	24 hr	48 hr
0.04	3/6	1/6
0.1	5/6	2/6
0.2	0/6	1/6
2.0	6/6	4/6

a. > control raw fever score + 2 SD

Summary, Part III:

To determine a FD₅₀ for SEB by the aerosol route, groups of monkeys were exposed to increasing total doses of SEB. Analysis of the data indicates that further work is required.

Publications:

None

ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 801: Mechanisms of Action of Microbial Toxins

Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland

Division: Physical Sciences

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Authors: David Auerbach, Captain, VC (I)
Theodore S. Herman, Captain, MC (II)

Reports Control Symbol: RCS-MEDDH-288 (R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
				DA OLO872	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8. DES'N INSTR'N	9. LEVEL OF SUM	
68 07 01	D. CHANGE	U	U	NA	DE	A. WORK UNIT	
10. NO./CODES:*		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62706A		1B662706A096		01	
b. CONTINGUOUS		62124011		1B622401A096		01	
c. CONTINGUOUS		CDOG 1212b(9)					
11. TITLE (Precede with Security Classification Code)*							
(U) Mechanisms of action of microbial toxins							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS*							
003500 Clinical Medicine; 004900 Defense; 003200 BW, CW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
66 10		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PREVIOUS		b. FUNDS (In thousands)	
b. NUMBER:*				69		3	
c. TYPE:				FISCAL YEAR		CURRENT	
d. KIND OF AWARD:				70			
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME:*				NAME:*			
USA Medical Research Institute of				Physical Sciences Division			
Infectious Diseases				USA Medical Research Institute of			
ADDRESS:*				ADDRESS:*			
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				Fort Detrick, Md 21701			
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301 663-4111 Ext 5233				301 663-4111 Ext 5214			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered.							
22. KEYWORDS (Precede EACH with Security Classification Code)				ASSOCIATE INVESTIGATORS			
(U) Enterotoxin; (U) Staphylococcus; (U) Coagulation; (U) Osmosis; (U) Vasopressin				NAME:			
				Herman, T. S.			
				DA			
23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Study the mechanisms of action of microbial toxins.							
24. (U) Hematologic and coagulative parameters are studied in animal hosts challenged with SEB. The effects of SEB on transepithelial transport processes are studied using as a model the permeability of the isolated toad bladder to the osmotic movement of water.							
25. (U) 68 07 - 69 06 - Monkeys, rabbits, and rats did not display disseminated intravascular coagulation in response to challenge with SEB. In preliminary studies, SEB did not affect the permeability to water of the isolated toad bladder.							

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B622706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 801: Mechanisms of Action of Microbial Toxins

Description:

To evaluate the mechanisms of action of microbial toxins.

Progress, Part I:

In 1966, Gilbert^{1/} reported a preliminary study on the coagulation mechanism and leukocyte response of beagle dogs to highly purified staphylococcal enterotoxin B (SEB) given by the intravenous (IV) route. He found that the most frequent and severe abnormalities occurred in 3 hr - \leq 4 days post challenge.

During the past year, we have studied the effects of an IV challenge of SEB on hematologic and coagulative parameters in 3 animal hosts: the monkey, a highly susceptible host; the less susceptible rabbit, and the rat - a highly resistant host. The parameters examined in each included total leukocyte and platelet counts, differential count, hematocrit, prothrombin and partial thromboplastin times, plasma fibrinogen and fibrinolysin.

In the monkey and rabbit, a marked neutropenia was noted within 5 min of injection which became most severe in 45-90 min. Thereafter, the neutrophil count gradually rose to marked neutrophilia at 12 hr, returning to normal in 24 hr. The lymphocytes, on the other hand, gradually decreased with the severest lymphopenia occurring in 24 hr. In the rat, the only change noted was a mild neutrophilia which peaked at 12 hr.

In contrast, the only coagulative change noted in the rabbit and monkey was a shortening of the time required for fibrinolysis which in the rabbit and monkey was shortened down to 20% of normal within 45-90 min. Thereafter a marked prolongation occurred with the longest time seen at 24 hr. Other parameters were unchanged. In the rat no alterations in coagulation were detected.

Thus, it appears that disseminated intravascular coagulation is not part of the mechanism of action of the toxin. However, in susceptible species, a circulating factor is activated which mediates the leukopenia; its significance is unknown.

Summary, Part I:

Disseminated intravascular coagulation due to intravenous challenge with SEB does not occur in the rhesus monkey or the rabbit.

Progress, Part II:

The isolated urinary bladder of the toad (Bufo marinus) is being employed as a mode system to study the effects SEB on transepithelial transport processes.

The method of Bentley^{2/} has been used to measure the osmotic movement of water. SEB at concentrations ranging from 0.025 µg/ml-2.5 µg/ml added to either the mucosal surface bathing solution or the serosal surface bathing solution for periods \leq 3 hr had no effect on the permeability of the bladder to water and did not significantly alter the stimulation of the osmotic flow of water produced by submaximal concentrations of vasopressin. Experiments are now underway which are designed to test the effect on water permeability of increasing the concentration of SEB and the duration of SEB treatment.

Summary, Part II:

SEB has not been found to have any effect on the permeability to water of the isolated urinary bladder of the toad under the experimental conditions employed.

Publications:

None

LITERATURE CITED

1. Gilbert, C. F. 1966. Effects of staphylococcal enterotoxin B on the coagulation mechanism and leukocytic response in beagle dogs - A preliminary study. *Thromb. Diath. Haemorrh.* 16: 697-706.
2. Bentley, P. J. 1958. The effects of neurohypophyseal extracts on water transfer across the wall of the isolated urinary bladder of the toad Bufo marinus. *J. Endocrinol.* 17: 201-209.

ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 802: In Vivo Distribution of Microbial Toxins

Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland

Division: Pathology

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Author: Hubert J. Wolfe, Major, MC

Reports Control Symbol: RCS-MEDDH-288(R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
				DA OL0873	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8. DISB'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62706A	1B662706A096	01	802			
b. CHALLENGE	62124011	1B622401A096	01				
c. CHALLENGE	CDOG 1212b(9)						
11. TITLE (Precede with Security Classification Code)*							
(U) In vivo distribution of microbial toxins							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS*							
003500 Clinical medicine; 004900 Defense; 003200 CW, BW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
66 10		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (In thousands)	
b. NUMBER: NA				FISCAL YEAR		69	
c. TYPE:				CURRENT		70	
e. KIND OF AWARD:						2	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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NAME: Crozier, D.				NAME: Wolfe, H. J.			
TELEPHONE: 301-663-4111 Ext 5233				TELEPHONE: 301 663-4111 Ext 6206			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence considered.				NAME:			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Enterotoxin; (U) Staphylococcus; (U) Immunofluorescence; (U) Immunoenzymatic techniques; (U) Isotopic tracers; (U) Antigen; (U) Antibody; (U) Horseradish peroxidase							
23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) Study the transport and localization of microbial toxins in animal tissues and the pathogenesis of the corresponding intoxications following various routes of challenge.							
24 (U) Enzyme tagging techniques for protein labeling are developed. Enzyme, fluorescein, and radioisotope tagged and untagged toxins are administered to susceptible species by various routes. Toxin distribution is determined by appropriate methods. Distributions noted are compared with similar studies using horseradish peroxidase (HRP), an innocuous protein of similar molecular weight.							
25 (U) 68 07 - 69 06 - For enzyme labeling of staphylococcal enterotoxin B (SEB) and its antisera, the diisocyanates proved to be the optimal conjugating agent and HRP the most effective tracer.							
<p>With the intravenous injection of SEB or HRP in the nonimmune susceptible animal, similar rates of removal from the blood were noted as was predominate renal convoluted tubular localization. Following intrabronchial introduction, however, SEB and HRP were handled differently. Three to 4 hr after exposure a rapid rise in the serum levels of SEB began to develop but a parallel rise of serum HRP failed to develop. However, in the latter cases the regional pulmonary lymph nodes contained abundant HRP deposits suggesting that normally the alveolar capillary membrane is impermeable to HRP and the lymphatic system is a major avenue of its removal. The evolution of HRP serum levels in SEB intoxicants resembled that described for SEB alone, and in these cases there was an absence of the predominant lymphatic localization. It was suggested that under these circumstances HRP is removed by another route, possibly by transport directly across the alveolar capillary membrane secondary to altered alveolar permeability.</p>							
Publication: Lab. Invest. 20:17-25, 1969.							

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 802: In Vivo Distribution of Microbial Toxins

Description:

Study the transport and localization of microbial toxins in animal tissues and the pathogenesis of the corresponding intoxications following various routes of challenge.

Progress:

Enzyme tagging techniques for proteins were investigated employing carbodiimide, dehalogenated dinitrobenzenes and diisocyanates as bifunctional agents and galactose oxidase, glucose oxidase and horseradish peroxidase (HRP) as enzyme tags. Staphylococcal enterotoxin B (SEB) and SEB antisera were employed as the protein model for labelling. Optimal histochemical techniques for the demonstration of the enzymes noted above in gel and cellulose acetate media were developed and efficacy of various bifunctional agents and enzymes in labelling was studied. The conjugated SEB or SEB antisera were reacted with a double diffusion system in titrated serial dilutions to study immunoprecipitin activity. Immunoprecipitin reactions were identifiable by either protein staining or histochemical demonstration of enzyme activity. Enzyme activity proved to be the most sensitive indicator. Hemagglutinin (HA) titers of SEB antisera were also determined after conjugation. The most effective conjugating agent proved to be the diisocyanates; and HRP was the most efficient of the enzyme tags employed.

Following intraduodenal, intrabronchial or intravenous (IV) administration of ^{125}I labelled and unlabelled SEB in SEB negative rabbits and rhesus monkeys, serial peripheral blood samples were drawn at 15-min intervals for 8 hr; SEB levels were measured by either quantitative radial immunodiffusion or trichloroacetic acid (TCA) precipitable ^{125}I activity. Results were compared with similar studies employing HRP, an innocuous protein of similar molecular weight. Following IV injection, the rate of removal of HRP from the blood compares favorably with that reported previously for SEB in the nonimmune susceptible animal. The predominant renal convoluted tubular localization reported with SEB by fluorochrome labelling was noted with HRP either by histochemical staining reactions or by autoradiographs of ^{125}I -labelled HRP. Introduction of SEB via the digestive tract failed to produce a consistent measurable rise in serum SEB levels or to develop significant renal localization. Following introduction into the bronchopulmonary tree a striking difference was noted in the handling of these 2 low molecular weight proteins.

Three hours after SEB exposure detectable serum levels were noted and were followed by a sharp rise in SEB serum levels over the following 5 hr. This was contrasted with the HRP which failed at any point during the 8-hr period to produce significant serum levels. In addition following intratracheal HRP instillation the regional pulmonary lymph nodes contained abundant HRP deposits, demonstrating that the lymphatic system is a major avenue of removal of this protein. This may be contrasted to SEB which fails to demonstrate such a pattern. It would appear to be transported by another route, possibly directly across the alveolar capillary membrane and into the circulation. Renal convoluted tubular localization of SEB was noted at time of sacrifice, 8 hr after intrabronchial instillation.

Following the intratracheal introduction of ^{125}I -HRP with noniodinated SEB, the regional lymph nodes were found to have no localization of ^{125}I -HRP, as noted above; blood levels of TCA precipitable ^{125}I activity rose in a manner similar to that observed with ^{125}I -SEB alone, suggesting under these circumstances that there was removal of the HRP by another route possibly by transport across an alveolar capillary membrane secondary to altered permeability. Intravenous injection of SEB following intratracheal HRP instillation also was associated with an elevation in serum HRP levels. This occurred within the first hour, much earlier in the course of exposure than that noted with combined intratracheal SEB and HRP instillation.

Summary:

For the enzyme labeling of SEB and SEB antisera, the diisocynates proved to be the optimal conjugating agent and horseradish peroxidase the most effective tracer.

With IV injection of SEB or horseradish peroxidase (HRP) in the nonimmune susceptible animal, similar rates of removal from the blood were noted as was renal convoluted tubular localization. Following intrabronchial introduction, however, SEB and HRP were handled differently. Three to four hours after exposure a rapid rise in the serum levels of SEB began to develop in the SEB intoxicants but a parallel rise of serum HRP did not occur in the animals given HRP. However, in the latter cases the regional pulmonary lymph nodes contained abundant HRP deposits suggesting that normally the alveolar capillary membrane is impermeable to HRP and the lymphatic system is a major avenue of its removal. The evolution of HRP serum levels in SEB intoxicants, however, resembled that described for SEB alone, and in these cases there was an absence of the predominant lymphatic localization. It is suggested that under these circumstances HRP is removed by another route, possibly by transport directly across the alveolar capillary membrane secondary to altered alveolar permeability.

Publication:

1. Normann, S.J., R.F. Jaeger, and R.T. Johnsey. 1969. Pathology of experimental enterotoxemia. The in vivo localization of staphylococcal enterotoxin B. Lab. Invest. 20:17-25.

ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 002: Evaluation of Efficacy of Experimental Vaccines

Reporting Installation: U. S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

Divisions: Medical, Virology and Animal Assessment

Period Covered by Report: 1 July 1968 to 30 June 1969

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Report Control Symbol: RCS-MEDDH-288(R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OLO829	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8A. DISSEM INSTR ^a	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES: ^a	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY	62706A	1B662706A096		02		002	
b. CONTRIBUTOR ^a	62124011	1B622401A096		02			
c. CONTRIBUTOR ^a	CDOG 1212b(9)						
11. TITLE (Precede with Security Classification Code) ^a							
(U) Evaluation of efficacy of experimental vaccines							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
003500 Clinical Medicine; 004900 Defense; 003200 BW, CW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
61 10		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: ^a				FISCAL		69	
c. TYPE:				YEAR		CURRENT	
d. KIND OF AWARD:				70		2	
e. AMOUNT:						235	
f. CUM. AMT.						235	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a USA Medical Research Institute of Infectious Diseases				NAME: ^a Medical & Animal Assessment Divisions			
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21. GENERAL USE				ASSOCIATE INVESTIGATORS			
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22. KEY WORDS (Precede EACH with Security Classification Code)							
(U) Vaccines; (U) Immunization; (U) Encephalitis, equine (VEE, EEE, WEE); (U) Globulin; (U) Q fever; (U) Adenovirus							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Evaluate experimental vaccines developed by various contractors, organizations or other governmental agencies.							
24. (U) Test vaccines are given to experimental animals, and when considered safe, to volunteers.							
25. (U) 68 07 - 69 06 - Inactivated WEE vaccine (tissue culture origin) was tested in volunteers. It was found to be safe and of low reactogenicity. Three different dosage schedules are being evaluated. A combined EEE and WEE vaccine was tested in volunteers and was found to be safe and of low reactogenicity. Antigenicity will be evaluated following completion of serological test. VEE Immune Globulin, Human, was administered to volunteers to determine the appearance and persistence of circulating antibody and to determine the effect of the globulin on infection with attenuated VEE vaccine. Additional studies in at-risk personnel to determine optimum time of administration of a booster to EEE vaccine are in progress.							
Adenovirus vaccine, live, oral, type 7 was administered to 16 volunteers. It was found to be safe, and of low reactogenicity, but exhibited a low degree of infectivity. An oral dose of vaccine containing approximately 5 logs tissue culture median infectious doses resulted in infection in 68% of vaccinees.							
Seed stock of <u>Coxiella burnetii</u> (Henzerling strain, Phase I, MP-2) for future vaccine production was tested and found free of adventitious agents during animal and egg passage. Safety and efficacy of a formalinized staphylococcal enterotoxoid B continued to be evaluated in rhesus monkeys. The toxoid appears safe in the doses tested. It partially protected monkeys against the illness and lethal effects of toxin given by the aerosol and intravenous routes.							
Publications: Amer. J. Trop. Med. 1969 2 papers. In press.							

^a Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 002: Evaluation of Efficacy of Experimental Vaccines

Description:

Evaluate experimental vaccines developed by various organizations, contractors, or other governmental agencies.

Progress, Part I:

Evaluation of Inactivated Western equine encephalitis vaccine: Clinical and serological responses to Western equine encephalitis (WEE) vaccine, inactivated, tissue culture origin, Lot 1-1967, were evaluated in volunteers in 2 separate studies. In the first study (Project No. FY 69-3) subjects were divided into two groups of 7 and 8 each, designated Group I and II. Two subjects in each group served as controls. On day 0 each volunteer received 0.5 ml of vaccine subcutaneously in the deltoid region of the arm; the controls received 0.5 ml of isotonic sodium chloride for Injection, Sterile, U.S.P., in the same manner. On day 28 members of Group I received 0.5 ml of vaccine while those in Group II received 0.25 ml of vaccine. Each subject had frequent laboratory evaluation for white blood cells and differential count, hematocrits, platelet counts, serum glutamic oxalacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), direct and indirect serum bilirubin, blood urea nitrogen (BUN), and alkaline phosphatase. All patients were observed daily following the initial dose of vaccine for 12 days for local and systemic reactions. After subsequent doses volunteers were observed at 24 and 48 hr post vaccination on an outpatient basis. Blood was drawn to obtain serum for neutralization test prior to immunization on days 14, 28, 42, 56, 90, 180 and 270 after the initial dose of vaccine; a final serum sample will be obtained on day 360. Systemic and local reactions are shown in Table I. No meaningful changes in clinical laboratory values were observed during the period of the study.

Serum log neutralization indices (LNI) were determined by the constant serum varying-virus method. One to 3-day-old suckling mice were inoculated via the intracerebral route with equal mixtures of pre- or postvaccination serum and dilutions of California strain WEE virus. Animals were observed for deaths for 10 days. Fifty percent end points were determined by the method of Reed and Muench.^{1/} The LNI is expressed as the difference in virus titer in the presence of pre- and postvaccination sera: As indicated in Table II, none of the 15 subjects had detectable neutralizing antibody prior to vaccination. Twenty-eight days after the first dose of vaccine 9 out of 15 subjects responded with LNI's ≥ 1.7 logs. Fourteen days following the second inoculation all subjects developed significant neutralizing antibody

(LNI ≥ 1.7). No meaningful difference in mean serological response was observed between the group which received the second dose of 0.5 ml and that which received the 0.25 ml dose. Additional serological data are not yet available.

TABLE I. REACTOGENICITY OF WEE VACCINE, INACTIVATED, TISSUE CULTURE ORIGIN, LOT 1-1967, IN 15 NON-EXPERIENCED SUBJECTS.

DOSE	(DAY)	REACTION	
		None	Mild
0.5 ml	(0)	8	7 ^a /
0.5 ml	(28)	6	2 ^b /
0.25 ml	(28)	4	3 ^c /

- a. 4 with mild headache, muscle soreness, and fatigue. 3 with tenderness at site of injection of < 12 hr duration.
- b. Local tenderness at site of injection of < 12 hr duration.
- c. 2 with mild occipital headache for < 12 hr duration. 1 with local soreness of 24 hrs duration.

TABLE II. NEUTRALIZING ANTIBODY RESPONSES IN WEE NON-EXPERIENCED SUBJECTS ADMINISTERED 2 DOSES OF WEE VACCINE, INACTIVATED, TISSUE CULTURE ORIGIN, LOT 1-1967.

LNI ^a /	NUMBER RESPONDING BY DOSE:				
	Control	Group 1		Group 2	
		0.5 ml	0.5 ml	0.5 ml	0.25 ml
3.0 - 3.9	0				1
2.0 - 2.9	0	1	2	7	5
1.7 - 1.9	0	4	2	1	1
1.0 - 1.6	0	3	3		
< 1.0					

- a. Log₁₀ serum neutralizing index of serum obtained 28 days after first dose of vaccine in 14 days after second dose of vaccine.

In the second study (Project No. FY 69-4) 6 volunteers not previously immunized with WEE vaccine and having no history of infection with the virus were administered 2 doses of 0.25 ml of WEE vaccine 28 days apart. Subjects were observed 24 and 48 hr after administration of each dose of vaccine for local and systemic reactions. Blood was drawn to obtain serum for neutralizing antibody prior to immunization and on days 7, 14, 28, 42, 56 and 90; additional samples will be obtained on days 180, 270 and 360 after the initial dose of vaccine. No local or systemic reactions developed. Serological data are not yet available.

Summary, Part I:

WEE vaccine administered to a limited number of subjects was found to be safe and of low reactogenicity. By 14 days following the second dose of vaccine all subjects had significant neutralizing antibodies (≥ 1.7). No significant difference in serological response was observed between the group which received the second dose of 0.5 ml and that which received the 0.25 ml dose.

Progress, Part II:

Evaluation of Combined WEE and Eastern equine encephalitis (EEE) Vaccine (Project No. FY 69-6). Clinical and serological responses were evaluated in 16 volunteers administered Eastern equine encephalitis (EEE) vaccine, inactivated, tissue culture origin, Lot 1-1966, and WEE vaccine, inactivated, tissue culture origin, Lot 1-1967 when administered in a combined form. The vaccine was reconstituted for injection by the addition of 5.5 ml Water for Injection, Sterile, U.S.P., to a vial of lyophilized EEE vaccine. Five ml of EEE vaccine were then withdrawn and added to a vial of lyophilized WEE vaccine and mixed. Each 0.5 ml of combined product contained approximately 100 hamster intraperitoneal median immunizing doses (GPIPID₅₀) of each vaccine. Each subject was administered 2 doses of 0.5 ml each of combined vaccine 28 days apart. Four volunteers served as controls and received 0.5 ml of isotonic sodium chloride of Injection, Sterile, U.S.P. in the same manner. Each subject had frequent clinical laboratory examinations as reported in Part I. Electrocardiograms (ECG) were obtained on each subject daily under standard conditions. Blood was drawn for neutralizing antibody prior to immunization and on days 14, 28, 42, 56; additional specimens will be obtained on days 90, 180, 270 and 360 days after the initial dose of vaccine.

Five vaccinees had mild nonspecific ST-T wave changes on serial ECG. The changes consisted of decreased amplitude, flattening and inversion of the T wave; they were confined to the inferior standard and lateral precordial leads. One control who developed an upper respiratory illness during the study had similar changes. All ECG returned to normal prior to the discharge. There was no evidence of heart disease during the period of study.

Forty-eight and 72 hr following vaccination, neutropenia occurred characterized by a total mean neutrophile count of 2556 and 2523 neutrophiles ($p < .001$) respectively. Only at 72 hr did a significant ($p < .05$) leukopenia occur. No other meaningful changes in clinical laboratory values occurred. Local and systemic reactions are shown in Table III. Serological data are not yet available.

TABLE III. REACTOGENICITY OF WEE VACCINE, INACTIVATED, TISSUE CULTURE ORIGIN, LOT 1-1967 AND EEE VACCINE, INACTIVATED, TISSUE CULTURE ORIGIN, LOT 1-1966 ADMINISTERED IN COMBINED FORM IN 16 NON-EXPERIENCED SUBJECTS

DOSE	(DAY)	REACTIONS ^{a/}							
		Local				Systemic			
		None	Minimal	Mild	Moderate	None	Minimal	Mild	Moderate
0.5 ml	(0)	12	2	1	1	8	5	2	1
0.5 ml	(28)	14		2		14		2	

a. Local:

- Minimal - discomfort and/or tenderness, < 24 hr duration.
- Mild - discomfort at site of injection plus tenderness, \geq 24 hr.
- Moderate - discomfort, tenderness, erythema, induration.
- Severe - marked swelling, fluctuation, necrosis.

Systemic:

- Minimal - Subjective complaints, < 24 hr duration.
- Mild - Subjective complaints, \geq 24 hr duration.
- Moderate - Rectal temperature 101-102 F, ambulatory.
- Severe - Rectal temperature \geq 102 F, not ambulatory.

Summary, Part II:

Combined EEE and WEE vaccine was administered to 16 volunteers. The vaccine was found to be safe and of low reactogenicity. Mild electrocardiographic changes were noted in 5 vaccinees and in one control. Forty-eight and 72 hours following vaccination neutropenia occurred.

Progress, Part III:

Evaluation of Venezuelan equine encephalomyelitis - Immune Globulin, Human (VEE-IG) (Project No. FY 69-5). VEE-IG Lot No. 0750D030 A1 was prepared by Hyland Laboratories, Los Angeles, California. It is identical with Immune Serum Globulin (Human) U.S.P., except that it was prepared from the plasma of donors specifically immunized with the attenuated VEE vaccine. Twenty-four healthy normal volunteers were administered VEE-IG intramuscularly to define the appearance and persistence of the antibody in the circulation and to determine the effect of this globulin on infections with attenuated VEE vaccine; serological data are not yet available.

Summary, Part III:

VEE-IG was administered intramuscularly to volunteers to determine the appearance and persistence of circulating antibody and to determine the effect of the globulin on infections with attenuated VEE vaccine. Serological data are not yet available.

Progress, Part IV:

Evaluation of Adenovirus Vaccine, Live, Oral, Type 7, Lot 16 CV-0100 (L-AV-7). The clinical and serological responses, safety, infectivity and pharyngeal and rectal shedding of L-AV-7 were evaluated in volunteers (Project No. FY 68-9).

The vaccine virus used for immunization was an adenovirus type 7, (strain 55142) propagated in human embryonic kidney (HEK) cells obtained by Wyeth Laboratories from Dr. Robert Chanock of National Institute of Allergy and Infectious Disease, Bethesda, Maryland. The strain was passaged 3 times in HEK, then 12 times in human diploid fibroblast cultures (WI-38); the product was then lyophilized, mixed with an inert vehicle, and distributed into enteric coated capsules (Lot 16 CV-01001, Wyeth). The capsules were shown to contain an average of $10^{4.9}$ tissue culture median infectious dose (TCID₅₀) when titrated in HEK cell cultures.

Twenty-four healthy volunteers found to be free of demonstrable adenovirus type 7 antibody by tissue culture neutralization test were studied. They were divided into 2 groups designated Groups A and B. Group A consisted of 16 volunteers who were fed $10^{4.9}$ TCID₅₀ of L-AV-7. Group B consisted of 8 unvaccinated subjects who were administered enteric coated placebo capsules (enteric coated press coat tablet Lot 350A-T-138, Wyeth); this preparation was shown to contain no cytopathogenic agent when a liquid suspension of it was inoculated into HEK tissue culture tubes. Volunteers were housed on 2 closed wards; each ward contained volunteers who received L-AV-7 and volunteers who received the placebo. All subjects were examined twice daily for 21 days following exposure for evidence of respiratory disease or other untoward reactions. Each subject had frequent laboratory evaluation for white blood cell and differential counts, hematocrit, platelet counts, SGOT, SGPT, direct and indirect serum bilirubin, BUN and alkaline phosphatase determinations. The above tests were performed according to standard laboratory procedures.

Blood was obtained on days -5, 0, 7, 10, 14, 18, 21 and 35 for serological studies. Serum neutralization tests were performed on serum samples from volunteers using HEK tube cultures and ADV Type 7 strain (A-3-143) virus. Serum neutralization end-points were determined at a time when the test dose of virus showed 100 TCID₅₀ in HEK tube cultures. Adenovirus complement-fixation titers on 0, 21, 35 day serum samples were determined by standard microtiter procedures against an antigen prepared against ADV Type 4 strain RI-67.

Throat washings and stool (or rectal swab) specimens were obtained from each volunteers on study days -4, -3, -2, and days 0 through 21; 0.3 ml portions of each throat washing and 0.3 ml portions of a 10% suspension of each stool specimen were inoculated into 3 HEK tube cultures. On study days -4, -3, -2 portion of throat washings were inoculated into WI-38 and rhesus monkey kidney tube cultures in addition to the HEK cells. Tubes were incubated at 36 C and observed for cytopathic effect (CPE) every other day. Isolates exhibiting characteristic ADV CPE were typed in tissue culture neutralization tests using HEK tube cultures and hyperimmune ADV Type 7 antiserum; those exhibiting typical herpes virus CPE were typed with hyperimmune Herpesvirus hominis antiserum.

No abnormalities in hematocrit, complete blood count, platelet count, total direct and indirect bilirubin, SGOT, SGPT, alkaline phosphatase, BUN or urinalysis were found in any volunteers in the immune or placebo groups during the duration of the study.

ADV-7 nasopharyngeal excretion was not demonstrated in throat washings of either immunized or placebo volunteers. Excretion of Herpesvirus hominis was demonstrated in throat washings of 4 volunteers, all in the L-AV-7 immunized group.

The pattern of virus excretion in the stool is shown in Table IV. Eleven of the 16 volunteers receiving L-AV-7 excreted ADV Type 7 in the stools. ADV-7 shedding was demonstrated first on study day 2 and last on study day 22. Duration of fecal shedding varied from 8 - 18 days with a mean of 13 days. None of the 8 volunteers receiving the placebo tablet excreted ADV in the stool during the study. All ADV isolated were typable as ADV Type 7; no virus other than ADV-7 was isolated from stool specimens of both immunized and placebo volunteers.

TABLE IV. ADENOVIRUS EXCRETION IN STOOL IN SUBJECTS RECEIVING L-AV-7^a/

VOLUNTEER NUMBER	ONSET OF RECTAL SHEDDING (Day of Study)	DURATION OF EXCRETION (Days)
1	6	12
3	5	9
6	3	15
9	2	8
12	5	14
13	6	14
17	4	17
18	4	18
20	6	16
21	8	8
23	4	9

a. No ADV-7 stool excretion was found in the placebo group.

Table V is a summary of the ADV-7 neutralization titers of volunteers, (immunized and placebo) on days 21 and 35 after immunization. Neutralizing antibody was not detected in the sera of the 8 placebo subjects or the 5 immunized volunteers who failed to excrete ADV-7. Antibody responses of the 11 ADV-7 immunized excretors were minimal; by day 35 two showed no detectable neutralizing antibody at a 1 to 2 serum dilution and maximal neutralization titers were 1:16.

TABLE V. ADV-7 NEUTRALIZING ANTIBODY TITERS IN SUBJECTS ADMINISTERED ORAL L-AV-7

	VOLUNTEER	DAYS ADV-7 EXCRETION	NEUTRALIZATION TITER ^a / (Reciprocal)		
			0 Day	21 Day	35 Day
<u>Immunized</u>					
Excretors ADV-7	1	12	<2	16	16
	3	8	<2	32	16
	6	15	<2	4	4
	9	8	<2	<2	<2
	12	11	<2	2	2
	13	14	<2	<2	<2
	17	15	<2	<2	8
	18	17	<2	<2	4
	20	16	<2	8	4
	21	8	<2	8	8
	23	9	<2	16	8
Non-Excretors	4	0	<2	<2	<2
	7	0	<2	<2	<2
	10	0	<2	<2	<2
	15	0	<2	<2	<2
	24	0	<2	<2	<2
<u>Placebo</u>					
	2	0	<2	<2	<2
	5	0	<2	<2	<2
	8	0	<2	<2	<2
	11	0	<2	<2	<2
	14	0	<2	<2	<2
	16	0	<2	<2	<2
	19	0	<2	<2	<2
	22	0	<2	<2	<2

a. Against 100 TCID₅₀ strain A-3-143

Mild afebrile upper respiratory disease was noted in 2 immunized and 1 control volunteer. In all 3 instances, the symptoms could not be associated with pharyngeal or stool ADV-7 excretion.

Four immunized volunteers developed diarrheal illness, one with fever and a rectal temperature of 102 F. As shown in Table VI, in volunteer no. 15 diarrhea was not associated with ADV-7 shedding. In 3 patients diarrhea was associated with ADV-7 stool excretion, although in volunteer no. 21 illness was first manifested 7 days after ADV-7 excretion began and 2 days before virus excretion ceased. No other viral pathogens were isolated from these volunteers. Thus, it is difficult to associate all 4 diarrheal episodes with ADV-7 infection, although an association of vaccine ADV-7 with disease in volunteers 17 and 20 cannot be eliminated. No other illnesses were detected in any of the immunized or placebo volunteers during the study.

TABLE VI. RELATIONSHIP BETWEEN ADV-7 RECTAL SHEDDING AND DIARRHEA IN IMMUNIZED SUBJECTS.

VOLUNTEER NUMBER	STOOL EXCRETION ADV-7 (Study Days)	DIARRHEA (Study Days)
15	NONE	2-4
17	4-17, 20	3
20	9-21	9-13
21	8-15	14-16

Since only 11 of 16 volunteers developed ADV-7 neutralizing antibodies, the infection rate with this lot of vaccine virus appears too low for use in man.

The pattern of stool ADV-7 excretion in volunteers infected with L-AV-7 was found comparable to that of ADV-4 vaccine virus stool excretion.⁴

No evidence of communicability of the vaccine virus was found; 8 placebo volunteers who lacked detectable serum ADV-7 neutralizing antibody and who were housed with the immunized group showed no virus excretion and did not develop neutralizing antibody rises during the course of the study. Serum neutralizing antibody titers were low with 2 of 11 ADV-7 excretors lacking demonstrable neutralizing antibody at a 1:2 dilution.

Summary, Part IV:

Adenovirus vaccine, live, oral, type 7, Lot 16 CV-0100 (L-AV-7) was administered to 16 volunteers. It was found to be safe and of low reactogenicity, but exhibited a low degree of infectivity. Eleven of 16 vaccinees excreted virus in the stool. No pharyngeal excretion of ADV-7 was observed in any of these volunteers. No evidence for person to person transmission of vaccine virus was obtained.

Approximately 68% of vaccinees developed infection when administered an oral dose of approximately $10^{5.0}$ TCID₅₀.

Progress, Part V:

The antigenic phase of the seed material Coxiella burneti (Henzerling strain),^{2/} was tested to verify that the antigen had remained predominantly Phase I.

The passage history of the seed material is as follows:

1. Henzerling isolate.
2. Six guinea pig passages.
3. Twenty-two egg passages.
4. One guinea pig passage, USAMRIID.
5. Four egg passages, WRAIR, RIF-free eggs.
6. One guinea pig passage, USAMRIID, guinea pig serologically negative for murine viruses.
7. Two egg passages, WRAIR, RIF-free eggs.

Twenty per cent yolk sac slurry of the 2nd egg passage material was titrated by various methods. Findings were $10^{4.8}$ median lethal doses (LD₅₀)/ml in embryonated eggs (WRAIR), 10^2 guinea pig intraperitoneal (GPIP) LD₅₀/ml, $10^{9.3}$ GPIP median fever dose (FD₅₀)/ml (fever = temperature ≥ 105 F), and $10^{10.5}$ GPIP median complement fixation titer (CF₅₀) (28th and 42nd day, Phase II - CF₅₀).

Guinea pigs inoculated with 10^{-3} and 10^{-4} dilutions of the seed material remained seronegative to the following adventitious viruses 30 days post-inoculation: PVM, Reo3, GDVII, Polyoma, Sendai, KRV, H-1, SV₅, Mouse Adeno, Mouse Hepatitis and LCM.

RIF-free chickens obtained from the National Institute of Health (NIH), Bethesda, Maryland, were inoculated with 0.5 ml of seed material. Serum samples were collected 1, 2, and 3 months postinoculation and forwarded to Dr. Nicola M. Tauraso, NIH, for testing for avian leukosis viruses (ALV) and ALV antibody. ALV and ALV antibody could not be detected in the sera by RIF, complement fixation for avian leukosis, or fluorescent antibody tests.^{3/}

Summary, Part V:

A quantity of C. burneti (Henzerling strain), 2nd egg passage material, was titrated for infectivity in guinea pigs and embryonated eggs; serologic testing indicates the material has remained free of adventitious agents. Second egg passage material contains the maximum amount of Phase I antigenic material, and will be used to prepare future stocks of vaccine.

Progress, Part VI:

The safety and efficacy of formalinized staphylococcal enterotoxoid B (SEB) was evaluated in rhesus monkeys (Macaca mulatta). The toxoid was produced under contract by Charles Pfizer Company, Inc., Terre Haute, Indiana. To date, data have been obtained on a small experimental lot (Lot 44 and 44D) and larger production lot (Lot 87285). The manufacturing processes used to prepare these lots are summarized in Table VII.

TABLE VII. MANUFACTURING PROCESS SUMMARY, DETOXIFICATION OF SEB

	LABORATORY Lot 44 & 44D ^{a/}	PRODUCTION Lot 87285
Production Date	December 1966	June 1968
Initial Toxin (SEB Lot 14-31R), mg	200	4,000
Diluent-Phosphate Buffer at pH 7.5, containing 0.8% Formalin, ml	100	2,000
Initial Concentration Toxoid, mg/ml	2	2
Detoxification at 37 C, days	30	40 ^{b/}
Final Concentration Toxoid, $\mu\text{g N}_2/\text{ml}$	260, 34	101
Concentration Thimerosal Final Product, $\mu\text{g}/\text{ml}$	100	49
Formalin Concentration Final Product, %	<0.002	0.016

a. Lots 44 and 44D differ only in concentration of μg nitrogen in the final production.

b. Detoxification was not complete at 30 days.

To establish a safety margin, monkeys were given doses of 500 and 1000 μg toxoid N_2 subcutaneously (the proposed vaccination dose of toxoid is 50 $\mu\text{g N}_2$). The monkeys were maintained in restraint chairs; rectal temperatures were continuously monitored by rectal thermocouple probes and a Honeywell Electronik (R) 15 Recorder (Honeywell, Inc., Lutherville, Maryland). The temperature response of each animal was plotted on graph paper containing 30 boxes per 1/2 in. All the boxes beneath the curve, and above a baseline representing 48 hr prevaccination, were added together and subtracted from the sum of the boxes under the curve for postvaccination. This number was termed the raw fever score. An equal number of monkeys were used as shams and were given phosphate buffer. An animal was called positive for fever if his raw fever score was greater than the mean + 2 SD of the raw fever scores of the sham vaccinated monkeys.

The results of the safety tests are shown in Table VII. Other than fever, no evidence of residual toxicity, such as emesis, diarrhea or death, was observed. No significant local reactions at the inoculation sites or typical hypersensitivity reactions, either at the time of the 2nd toxoid dose or at the time of challenge, were seen in these animals or those used in the protection studies.

TABLE VII. DETOXIFICATION (SAFETY) TESTS OF SEB TOXOID.

DAYS DETOXIFIED	DOSE µg N ₂	LAB LOT 44 & 44D	PRODUCTION LOT 87285	
		Rhesus ^a /	Rhesus ^a /	Cynomolgus ^b /
		Ill/Total	Ill/Total	Ill/Total
30	500	0/2	-	2/6 (Emesis)
	1,000	0/2	-	-
40	500	-	0/4 ^c / 0/4 ^d /	0/6
	1,000	-	2/8 1/2 ^d /	-
			(Fever only, no emesis)	-

- a. Performed by USAMRIID personnel.
- b. Performed at Charles Pfizer Company.
- c. SEB hemagglutination antibody negative.
- d. SEB hemagglutination antibody positive.

For the protection tests, SEB hemagglutinating antibody-negative rhesus monkeys weighing 2-4 kg were used. The various vaccination and challenge schedules used to date are shown in Table IX.

TABLE IX. SCHEDULE FOR VACCINATION AND CHALLENGE OF MONKEYS

VACCINE DAY	LOT	TOXOID DOSE µg Nitrogen	CHALLENGED Months ^a /
0, 28	44	80	1.5
0, 28	44	80	12.0
0, 28	44	34	1.5
0, 28	44	34	6-7
0, 28	44	17, 34	1.5
0, 28	87285	50	1.5

- a. Time after 2nd toxoid dose.

TABLE X. EFFICACY OF TWO DOSES STAPHYLOCOCCAL ENTEROTOXOID B IN MONKEYS CHALLENGED WITH SEB.

TOXOID DOSE μg N ₂ SC	CHALLENGE TIME Months	CHALLENGE DOSE ^a / AND ROUTE									
		10 μg/kg					≥ 300 μg/kg ^{b/}				
		IV		Aerosol		Dead/Total V C	IV		Aerosol		Dead/Total V C
		Ill/ V ^c	Total C ^c	Ill/ V	Total C		Ill/ V	Total C			
<u>Lot 44</u>											
80	1.5	1/13	13/13	1/8	8/8	6/13 ^{b/}	8/13 ^{b/}	-	-	-	-
80	12	0/3	3/3	-	-	0/2	3/3	-	-	-	-
34	1.5	1/8 ^{d/}	12/12 ^{d/}	2/6	6/6	2/10 ^{d/}	9/12 ^{d/}	0/6	5/6	5/6	5/6
34	6-7	1/6	9/9	-	-	3/6	9/9	-	-	-	-
17, 34	1.5	-	-	1/6	3/6	-	-	4/6	5/6	5/6	5/6
<u>Lot 87285</u>											
50	1.5	4/17	12/12	-	-	2/12	5/6	-	-	-	-

a. Lot 14-30, except 14-31R used for 34 μ g/kg dose at 1.5 months, see footnote d.

b. Five challenged with 1000 μ g/kg.

c. V = vaccinated; C = unvaccinated.

d. 3/8, 3/12, 3/10, 3/12

As can be seen in Table X, vaccination provided a high, though certainly not absolute, degree of protection against illness with intravenous (IV) or aerosol challenge of 10 $\mu\text{g/kg}$ SEB. In addition, those animals that did become ill appeared to recover much faster than the corresponding controls. The toxoid also provided some protection against the lethal effects of a 300 $\mu\text{g/kg}$ IV or aerosol challenge with SEB, as long as the toxoid dose was $\geq 34 \mu\text{g N}_2$ for both doses. Based on these results and the comparison of serological data, it was tentatively concluded that 2 doses of at least 34 $\mu\text{g N}_2$ /dose should be used for vaccination. Although the number of animals challenged at 6 months and 1 yr after vaccination is very small, the protection afforded by the toxoid does appear to be maintained for at least 6 months when animals received 2 doses of 34 $\mu\text{g N}_2$, and as long as 1 yr when they received 2 doses of 80 $\mu\text{g N}_2$.

To establish the storage life of the toxoid at 4 C, monkeys were vaccinated after the toxoid had been stored for 1 yr (Table XI). It appears that the toxoid retains its activity for 1 yr under these conditions.

TABLE XI. EFFICACY OF LOT 44 TOXOID AFTER STORAGE AT 4 C

AGE TOXOID (Lot 44)	TOXOID DOSE $\mu\text{g/N}_2$	CHALLENGE DOSE IV					
		10 $\mu\text{g/kg}$		300 $\mu\text{g/kg}$		1000 $\mu\text{g/kg}$	
		Ill/Total		Dead/Total		Dead/Total	
		va/	C	V	C	V	C
1 mon	80	0/5	5/5	-	-	3/5	4/5
13 mon	80	1/8	8/8	3/8	4/8	-	-

a. See footnote C Table VIII.

Summary, Part VI:

The safety and efficacy of a formalized SEB was evaluated in rhesus monkeys. The toxoid had a safety margin in monkeys at least 10 times the proposed vaccination dose. It partially protected against the illness and lethal effects produced by SEB toxin given by aerosol and IV routes.

Publications:

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JOD, DPG
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ANNUAL PROGRESS REPORT

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Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 003: Chemoprophylaxis and Therapy of Infectious Diseases of Potential Biological Warfare Significance

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Divisions: Medical and Physical Sciences with U. S. Food and Drug Administration

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23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Assess the effect of microbials and various drug regimens in various infectious diseases.							
24. (U) Various drugs are tested in volunteers under strict protocol conditions.							
25. (U) 68 07 - 69 06 - Publication of the chloramphenicol absorption study is anticipated following some revisions requested by the editor.							
Publication: Amer. J. Med. Sci. 1969. In press.							

^a Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 003: Chemoprophylaxis and Therapy of Infectious Diseases of Potential Biological Warfare Significance

Description:

Assess the effect of antimicrobials and various drug regimens in various diseases.

Progress and Summary:

A publication referring to a previous study^{1/} has been accepted for publication American Journal of the Medical Sciences, subject to some revision of the manuscript.

Publication:

1. Bartelloni, P. J., F. M. Calia, H. L. Ley, Jr., B. H. Minchew, and W. R. Beisel. 1969. Absorption and excretion of two chloramphenicol products in humans after oral administration. Amer. J. Med. Sci. In press.

LITERATURE CITED

1. U. S. Army Medical Unit. 1 July 1968. Annual Progress Report, FY 1968 p. 107 to 114. Fort Detrick, Maryland.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 004: Studies in Combined Antigens

Reporting Installation: U. S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

Division: Bacteriology

Period Covered by Report: 1 July 1968 to 30 June 1969

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Reports Control Symbol: RCS-MEDDH-288(R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a		2. DATE OF SUMMARY ^a		REPORT CONTROL SYMBOL	
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7. REGRADING ^a NA		8. DISSEM INSTR ^a DE		9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO		10. LEVEL OF SUM A. WORK UNIT			
10. NO./CODES: ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY		62706A		1B662706A096		02		004	
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19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION					
NAME: ^a USA Medical Research Institute of Infectious Diseases				NAME: ^a Bacteriology Division USA Medical Research Institute of Infectious Diseases					
ADDRESS: ^a Fort Detrick, Md 21701				ADDRESS: ^a Fort Detrick, Md 21701					
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)					
NAME: Crozier, D.				NAME: ^a Ward, M. K.					
TELEPHONE: 301 663-4111 Ext 5233				TELEPHONE: 301 663-4111 Ext 3246					
21. GENERAL USE				ASSOCIATE INVESTIGATORS					
Foreign intelligence considered.				NAME: Irwin, W. S.					
				NAME: Klein, P. A. DA					
22. KEYWORDS (Precede with Security Classification Code) ^a (U) Antigens; (U) Immunization; (U) Encephalitis, equine (VEE); (U) Tularemia; (U) Plague; (U) Vaccines; (U) Macrophages; (U) Tuberculin									
23. TECHNICAL OBJECTIVE. ^a 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23. (U) Determine the feasibility of combining various immunizing antigens; establish the best means to use such combinations and evaluate their immunogenicity.									
24. (U) Various antigens will be mixed in a variety of proportions and given to experimental animals for challenge studies. Promising combinations will be tested further and eventually may be tested in volunteers.									
25. (U) 68 07 - 69 06 - Mice simultaneously immunized with attenuated VEE (TC-83) and tularemia (LVS) vaccines show increased mortality over that observed with the vaccines singly given. Four to 7 days separation of them reduces the mortality rate. In guinea pigs and monkeys no deaths occur but there is also little enhancement of immunity. When guinea pigs are immunized with combined TC-83 + LVS + living plague vaccines there is a delayed but possibly increased antibody titer to plague. LVS has no adjuvant effect on responses to staphylococcal enterotoxin B toxoid in monkeys.									
TC-83 interferes with an <u>in vitro</u> test of delayed hypersensitivity to partially purified derivative of tuberculin (PPD) 1 week after infection. <u>In vivo</u> delayed hypersensitivity responses may be inhibited 2-4 months after infection.									
The effect of TC-83 immunization on antibody responses of guinea pigs to several purified, soluble antigens has been studied and published.									
Publications: J. Immun. 1969. In press (2 papers).									

*Available to contractors upon originator's approval.

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JOD, DPG

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 004: Studies in Combined Antigens

Description:

Determine the feasibility of combining various immunizing antigens; establish the best means to use such combinations and evaluate their immunogenicity.

Progress, Part I:

Vaccination of mice and a combination of attenuated Venezuelan Equine Encephalitis (VEE) (TC-83) and living tularemia vaccine (LVS) markedly increased mortality from vaccination alone.^{1/} Simultaneous vaccination in separate sites on the animal gave similar results. Animals that survive vaccination, however, are solidly protected against challenge with virulent Pasteurella tularensis and VEE virus, while about 15-20% of mice receiving LVS vaccine alone usually succumb to tularemia challenge.

These observations led us to examine the question of whether staggering the timing of the immunizations might reduce vaccination mortality and at the same time, give increased protection against tularemia challenge. In addition, during the course of the year the work with LVS-TC-83 vaccination and other combinations of vaccines was extended to guinea pigs and, in 2 cases, to monkeys.

The following vaccines and dosages were used singly and in combinations: (1) LVS: 3×10^4 - 1×10^5 organisms for mice, 1×10^8 for guinea pigs and monkeys; (2) TC-83: one standard human dose (approximately 1 to 5×10^3 guinea pig intraperitoneal infecting doses - GPIPID) in all cases; (3) EV 51 F strain of attenuated plague: 3×10^5 organisms; (4) SEB toxoid: 50 μ g toxoid N (monkeys only). Results of this work are summarized under appropriate sections below.

Staggered immunization with LVS and TC-83 vaccines. Mice: Groups of 20 animals were immunized with LVS at 7, 5, 4, 3, 2 and 1 days prior to and after TC-83, as well as simultaneously and alone.

Separation of the vaccines by at least 4-7 days reduced vaccination mortality from the combinations; in some instances, survival of the vaccines after virulent challenge appeared to be somewhat better than in the group

of animals given LVS alone. However, the only animals solidly protected against challenge were those in the group given the 2 vaccines simultaneously.

Serological studies revealed no differences in response among the groups. All sera were negative in tularemia agglutination tests prior to challenge; occasional sera from survivors showed titers of 1:20 or 1:40. Results of VEE hemagglutination-inhibition (HI) tests were similar to animals vaccinated with TC-83 alone.

Guinea pigs: Animals were vaccinated at the intervals described above for mice. There were no deaths from immunization in any group. There was good serological response to both vaccines. But none of the animals survived tularemia challenge, although time to death was prolonged beyond that of controls.

Monkeys: There were no untoward reactions to the vaccine combinations in this species. The serological response to LVS was identical in all groups. However, there appeared to be a significant depression of serological response to VEE in animals given LVS 1 day prior to TC-83. This experiment will be repeated as soon as sufficient numbers of animals are available for this study. None of the monkeys were challenged following immunization. It is hoped that a virulent challenge can be included in the next experimental protocol.

Combined TC-83, LVS and EV 51 F plague vaccines. Groups of guinea pigs were vaccinated with each of the vaccines separately and in combination. In one exploratory experiment, additional, similar groups were given the respective vaccines in a diluting fluid containing the preservatives (phenol or methiolate) used in typhoid, Q fever and tetanus toxoid vaccines, to determine the effect of these substances on the immunogenicity of the living vaccines, prior to work combining them with the killed vaccines indicated.

There were no deaths or other untoward effects from vaccination alone. The serological responses of the guinea pigs were not affected by the presence of preservatives in any case. Tularemia agglutinin titers and VEE HI antibody levels were identical in all animals given these vaccines in combination or alone, with or without preservatives.

There was evidence, however, that the peak plague hemagglutinating (HA) antibody response was definitely delayed in animals given the triple vaccine. Plague antibody titers of sera taken at 14 and 28 days from groups of 10 and 7 animals, respectively, in 2 different experiments are shown in Table I. As can be seen from the table, the mean titers of animals given plague alone had reached a peak at 14 days postimmunization. On the other hand, peak titers in animals given the triple vaccine were not reached until sometime between 14 and 28 days. In addition, titers in these animals at 28 days were slightly higher than in animals given plague vaccine alone. This observation will be explored further in later studies.

TABLE I. RESPONSE OF GUINEA PIGS TO VACCINATION WITH LIVING ATTENUATED PLAGUE VACCINE ALONE AND IN COMBINATION ^{a/}

RECIPROCAL PLAGUE HA TITERS BY DAY						
Plague Alone			Plague + TC-83 + LVS			
Experiment #1	Experiment #2		Experiment #1	Experiment #2		
14	28	14	28	14	28	28
128	64	256	128	4	256	< 4
256	128	256	128	16	256	64
128	64	128	256	< 4	512	32
64	128	128	64	< 4	512	16
256	256	256	64	16	128	< 4
256	256	512	1024	8	512	< 4
64	64	512	256	< 4	256	< 4
128	256	128	256	16	64	
16	128			8	256	
64	64			< 4	128	
Mean	136	140	272	272	287	16
Range	16-256	64-256	128-512	64-1024	64-512	<4-64

a. All control titers negative

All animals with any detectable HA antibody to plague survived a virulent challenge with approximately 100 LD₅₀ of virulent plague organisms.

LVS - SEB toxoid combination in monkeys. Several years ago, Greenberg et al²⁷ showed that LVS in combination with anthrax protective antigen (APA) markedly enhanced the protective efficacy of APA in guinea pigs. The possibility that LVS might have an "adjuvant" effect on staphylococcal enterotoxin B (SEB) toxoid in monkeys was explored.

A group of 12 monkeys was given the combination of LVS and SEB toxoid; their serological responses, as well as their responses to challenge with toxin, were compared with a similar group of animals given toxoid alone.

There was no detectable difference between the groups in either case. The toxoid used was fluid -- not alum precipitated, as had been the case with APA. The possibility that this difference in physical state of the non-living antigens used might account for lack of adjuvant action of LVS and will be explored as soon as alum-precipitated SEB toxoid becomes available.

Summary, Part I:

Simultaneous vaccination of mice with LVS and TC-83 always results in markedly increased vaccine mortality, but survivors are better protected against virulent tularemia challenge than animals given LVS alone. Separation of the 2 vaccines by 4-7 days reduces vaccine mortality, but gives only slightly increased protection. The same combinations in guinea pigs and monkeys do not give untoward reactions or death in these species, but there is also no enhancement of immunity to tularemia.

Mice rarely, if ever, develop a detectable serological response to LVS vaccination either alone or in combination with TC-83. Guinea pigs and monkeys, however, respond with relatively high titers of agglutinins to the single or combined vaccine.

Vaccination of guinea pigs with a combination of LVS, TC-83 and living attenuated plague delays the appearance of peak antibody titers to plague, but may enhance final antibody response.

LVS does not appear to have an adjuvant effect on response to SEB toxoid in monkeys.

Progress, Part II:

Studies on the effect of TC-83 on the immune response of guinea pigs have been extended to include delayed hypersensitivity-like reactions. The techniques used have been: the standard intradermal (ID) skin test, measurements of the swelling of the foot pad following intradermal injection,

and the in vitro migration of macrophages from a capillary by the technique of David et al.^{3/} Swelling of the foot was measured 24 hr after ID injection of the test antigen. The foot of the animal was immersed, to a tattooed line, in a beaker of mercury placed on a balance, then counter-balanced with water from a burette. The amount of water used to rebalance the balance is a measure of the size of the foot. Differences in measurements taken before and after injection reflect the degree of swelling, and thus the animal's response to the antigen.

David's technique uses oil-induced peritoneal exudates containing lymphocytes and macrophages. These cells are washed, packed in capillary tubes, and incubated 24 hr in small plastic petri dishes.

In control media of Eagles' minimal essential medium (MEM) with 15% normal guinea pig serum, the cells migrate from the end of the capillary tube over the bottom of the petri dish. Addition of various test agents to the media will inhibit cell migration to an extent dependent upon the sensitization of the cells and the concentration of the test agent.

Tracing an enlarged projection of the cell migration and subsequent planimetry allows quantitative measurements. Results are expressed as per cent migration of the cells exposed to a test substance, taking the migration of control cells as 100%. Data in the accompanying tubes represents the mean per cent migration from 4 capillaries in 2 petri dishes.

David and others^{3/} have shown that inhibition of macrophage migration by this technique correlates with in vivo measurements of delayed hypersensitivity. It should be noted that the purified protein derivative of tuberculin (PPD) used was the tablet form of PPD, which contains expecient. This form of PPD alone inhibits macrophage migration from normal animals.

All animals were vaccinated subcutaneously with 0.5 ml of TC-83 containing 1×10^3 GPIPID. The animals were injected in the foot pads and into the neck with 0.5 ml of a 1:1 mixture of complete Freund's adjuvant (CFA) and bovine gamma globulin (BGG) (2 mg/ml) 24 hr later.

Investigations have centered on 2 problems: the interference by previous exposure to TC-83 with the usual inhibition of macrophage migration observed with expecient-containing PPD, and evidence for an adjuvant effect of TC-83 on delayed hypersensitivity. Table II shows that vaccination with TC-83, 24-48 hr before exposure to PPD or complete Freund's adjuvant (CFA), significantly reduces the inhibition of macrophage migration by expecient-containing PPD when tested on day 7. This effect is no longer observed on day 14. The same phenomenon is not observed with BGG. Table III shows the results of experiments to determine whether exposure to TC-83 alone is responsible for the phenomenon. As can be seen, normal animals, animals receiving TC-83 alone, and animals immunized with CFA/BGG all respond similarly, but cells from animals receiving TC-83 before immunization with CFA/BGG are no longer inhibited by PPD.

TABLE II. EFFECT OF TC-83 VACCINATION ON GUINEA PIG MACROPHAGE MIGRATION IN VITRO

EXPOSURE OF ANIMALS	MEAN PER CENT MIGRATION COMPARED TO CELLS NOT EXPOSED TO ANTIGEN						NUMBER OF ANIMALS
	Day 7			Day 14		Day 21	
	PPD	BCG	PPD	BCG	PPD		
-24 hr	0						
--	CFA/BCG	53	-	35	-	25	3
TC-83	CFA/BCG	93	-	43	-	30	3
--	ICFA+BCG+PPD ^{a/}	42	85	53	88	-	4
TC-83	ICFA+BCG+PPD	72	74	48	74	-	4
--	PPD	23	-	87	-	84	5
TC-83	PPD	51	-	72	-	79	5
-48 hr	0						
--	CFA/BCG	57	-	31	35	-	5
TC-83	CFA/BCG	79	-	38	36	-	5

a. ICFA - Incomplete Freund's adjuvant.

TABLE III. SUMMARY OF TC-83 EFFECT ON MACROPHAGE MIGRATION AT ONE WEEK

EXPOSURE OF ANIMALS		% MIGRATION COMPARED TO CELLS NOT EXPOSED TO ANTIGEN ON DAY 7				ANIMALS/GROUP
		PPD		BGG		
		Mean	Range	Mean	Range	
-24 hr	0					
BSS	BSS	64	(43-78)	98	(69-131)	11
TC-83	BSS	67	(43-88)	101	(89-120)	6
BSS	CFA/BGG	61	(43-79)	92	(67-114)	8
TC-83	CFA/BGG	87	(57-123)	100	(86-113)	14

No such phenomenon is observed in the same animals when tested with BGG. Thus, the exposure to TC-83 followed by exposure to a PPD containing antigen, but neither VEE alone or antigen alone, alters the test system such that macrophages no longer respond by showing inhibition.

Table IV shows the results of testing groups of animals of 10 each by the foot pad swelling test. The size of the foot was calculated before exposure to antigen and 24 hr later. Batson's unpaired "t" test^{4/} was applied to the data to determine significance of the differences observed. At 1 week, animals receiving TC-83 before antigen did respond to PPD, but animals receiving antigen alone did not. These results are consistent with enhancement by VEE of the expression of delayed type hypersensitivity to PPD. These results are opposite to those observed with the in vitro system and may be influenced by antibody (vide infra).

Results of the in vitro experiment do not indicate whether the action of VEE at 7 days is an interference with the immunologic mechanism or a nonimmunologic alteration in the metabolism of the antigen. Current work is directed along 2 lines. Expecient-free PPD is being tested to determine the possible role of the impurities. Other experiments are underway to determine whether the macrophage or the lymphocyte is the primary cell involved preventing the action of PPD.

The failure of a delayed hypersensitivity response has been termed anergy and is well recognized in man following certain viral infections such as measles, in neoplastic diseases such as Hodgkin's disease, and in some diseases of unknown etiology such as sarcoidosis. Vaccination with TC-83 may serve as an animal model for the study of anergy.

TABLE IV. t VALUES FOR FOOT PAD SIZE 24 HOURS AFTER ANTIGEN INJECTION
COMPARED TO PREINJECTION VALUES

ANIMAL GROUP	TEST WITH	t VALUES WITH RESPECT TO PREINJECTION							
		Day 7		Day 21		Day 35		Day 49	
		t	P <	t	P <	t	P <	t	P <
Control	PPD	0.61	ns	0.94	ns	0.13	ns	0.30	ns
	BGG	0.56	ns	1.10	ns	0.35	ns	0.10	ns
TC-83 alone	PPD	1.00	ns	1.24	ns	0.40	ns	N.C. ^{a/}	0.90
	BGG	0.01	ns	0.66	ns	1.30	ns	0.30	0.77
CFA/BGG + TC-83	PPD	2.69	.05	2.06	ns	0.79	ns	N.C.	ns
	BGG	1.80	ns	4.23	.001	0.80	ns	.89	N.C.
CFA/BGG	PPD	1.10	ns	1.38	ns	1.29	ns	1.89	ns
	BGG	1.10	ns	4.05	.001	1.56	ns	2.57	2.35
									.05

a. N.C. = Not calculated. Mean foot pad size before and after injection remained essentially the same size.

Evidence for a long term adjuvant effect of TC-83 injection also has been contradictory by the 3 techniques employed. Table IV indicates a significant sensitivity to both PPD and BGG in animals receiving CFA/BGG alone at day 105 after immunization; animals receiving TC-83 + CFA/BGG alone do not show the same degree of sensitivity. The data presented in Table IV must be examined with 2 facts in mind: (1) the role of antibody in producing foot pad swelling is not defined; (2) the mechanics of the technique make it of less than optimal sensitivity. By skin test criteria, we know that most animals immunized with CFA/BGG are sensitive to both PPD and BGG on days 21 and 35 postimmunization. Because of the inertia in the mechanical balance used to make these measurements, only large differences will be manifest. Thus, the test is going to show large differences, but results do not necessarily correlate well with skin test sensitivity.

Table V presents values for skin tests in animals receiving TC-83 before antigen and animals receiving antigen alone. Animals immunized with incomplete Freund's adjuvant containing PPD and BGG after TC-83 had significantly larger skin induration than animals which did not receive the vaccine. No evidence of sensitivity was seen using PPD alone as the antigen.

TABLE V. EFFECT OF TC-83 ON SKIN TEST REACTIONS TO PPD

EXPOSURE OF ANIMALS			MEAN AREA OF INDURATION FOLLOWING PPD TEST BY DAYS					
			-24 hr	0	18	35	50	71
--	ICFA/BGG/PPD ^{a/}		34	41	10.4			26
TC-83	ICFA/BGG/PPD		36	59	38.0			99
					p < .05			p < .001
--	PPD						21	8
TC-83	PPD						29	25

a. ICFA = Incomplete Freund's adjuvant

Three of the above groups of animals were sacrificed between 105 and 140 days postimmunization and sensitivity to PPD and BGG was tested by macrophage migration. The only group in which an adjuvant effect of VEE was apparent is indicated in Table VI.

TABLE VI. MACROPHAGE MIGRATION ON DAY 140 AFTER IMMUNIZATION

EXPOSURE OF ANIMALS		PER CENT MIGRATION WHEN TESTED WITH	
-24 hr	0	PPD	BGG
--	CFA/BGG	39 \pm 12	37 \pm 11
VEE	CFA/BGG	17 \pm 7	53 \pm 10
t		p = <.05	ns

Summary, Part II:

TC-83 has been shown to interfere with the early expression of delayed hypersensitivity to PPD using an in vitro technique. This effect is dependent on prior immunization of the animal with PPD and TC-83. Studies are currently underway to determine the cellular site of action of this phenomenon and whether the action is immunologic or metabolic. In vivo studies indicate an enhancement by TC-83 of the animal's response to antigen, but the role of antibody in this response is not defined.

TC-83 does occasionally have an adjuvant effect on delayed hypersensitivity responses to PPD in guinea pigs, as determined by in vitro tests. This effect is best demonstrated 3-4 months after exposure to the vaccine and antigen. By in vivo testing the opposite is true. Again, the role of antibody is not defined.

Progress, Part III:

Studies on the effect of vaccination with the living attenuated strain of VEE (TC-83) on antibody response to several purified, soluble antigens in guinea pigs and mice have been completed. Results of this work are summarized in the two publications listed.

Presentation:

1. Howard, R. J., C. P. Craig, W. A. Hook, and S. E. Mergenhagen. Enhanced humoral immunity in mice injected with attenuated Venezuelan equine encephalitis virus. Presented at Federation of American Societies for Experimental Biology. 13-18 April 1969.

Publications:

1. Craig, C. P., S. L. Reynolds, J. W. Airhart, and E. V. Staab. 1969. Alterations in immune responses by attenuated Venezuelan equine encephalitis vaccine. I. Adjuvant effect of VEE injection in guinea pigs. J. Immun. In press.

2. Airhart, J. W., G. S. Trevino, and C. P. Craig. 1969. Alterations in immune responses by attenuated Venezuelan equine encephalitis vaccine. II. Pathology and soluble antigen localization in guinea pigs. J. Immun. In press.

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1. U. S. Army Medical Unit. 1 July 1968. Annual Progress Report, FY 1968. p. 115 to 122. Fort Detrick, Maryland.

2. U. S. Army Medical Unit. 1 July 1964. Annual Progress Report, FY 1964. p. 165 to 176. Fort Detrick, Maryland.

3. David, J. R., S. Al-Askari, H. S. Lawrence, and L. Thomas. 1964. Delayed hypersensitivity in vitro. I. The specificity of inhibition of cell migration by antigens. J. Immun. 93:264-273.

4. Batson, H. C. 1956. An introduction to statistics in the medical sciences. p. 18 to 20. Burgess Publishing Co., Minneapolis, Minn.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties.

Work Unit No. 096 02 005: Studies on Binding Properties of Antibodies

Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland

Division: Bacteriology

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Author: Mary H. Wilkie, M.S.

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10. NO./CODES: ^a		PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY		62706A	1B662706A096	02		005	
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a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
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NAME: ^a USA Medical Research Institute of Infectious Diseases ADDRESS: ^a Fort Detrick, Md 21701				NAME: ^a Bacteriology Division USA Medical Research Institute of Infectious Diseases Fort Detrick, Md 21701 PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution) NAME: ^a Wilkie, M. H. TELEPHONE: 301 663-4111 Ext 6130 SOCIAL SECURITY ACCOUNT NUMBER:			
RESPONSIBLE INDIVIDUAL NAME: Crozier, D. TELEPHONE: 301 663-4111 Ext 5233				ASSOCIATE INVESTIGATORS NAME: NAME: DA			
21. GENERAL USE Foreign intelligence considered.							
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Antigens; (U) Antibody; (U) Binding strength; (U) Serology							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23. (U) Study antigen binding properties of antibodies as a means of assessment of protective efficacy of experimental vaccines and therapeutic antisera against bacterial and viral agents. 24. (U) Establish an arbitrary scale to express binding affinities in place of presently used equilibrium constants, utilizing different biological or physical chemical techniques. Results will be related to protection tests. 25. (U) 68 07 - 69 06 - In sera from early bleedings of groups of rabbits hyperimmunized with bovine serum albumin (BSA) in 3 different physical states, binding capacity was greater than precipitin activity, but precipitins rose to equal binding levels in later bleedings. BSA in Freund's adjuvant stimulated much higher, more sustained total quantities of antibody than did soluble or alum precipitated BSA during the 60-day period following immunization. The anamnestic response to boosters a year later indicated that all 3 groups had been equally primed by the initial immunizations. Therefore, measurements of serological response during the 30-60 days following a primary immunization series may not reflect the true immune state of the animal. It was found that degradation of BSA by pronase is markedly inhibited (up to 95%) after combination of the antigen with antibody. The extent of the protection against enzyme hydrolysis afforded by different BSA antisera was roughly proportional to their binding capacity.							

^a Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 005: Studies on Binding Properties of Antibodies

Description:

Study antigen binding properties of antibodies as a means of assessment of protective efficacy of experimental vaccines or therapeutic antisera against bacterial and viral agents.

Progress:

Using bovine serum albumin (BSA) as the model antigen and rabbit anti-BSA sera, experiments are continuing on the evaluation of measurements of antibody binding capacity. The total binding capacity of antisera was determined by the Farr technique,^{1/} modified for the use of micro amounts of reagents. A modification of the method of Hunter and Greenwood^{2/} reagents was used to prepare ¹³¹I-labelled BSA. This procedure was found to give 85-95% iodination efficiency when protein concentrations exceeded 10 mg/ml.

Sequential bleedings from rabbits hyperimmunized with BSA in Freund's adjuvant or with soluble or alum-precipitated BSA have been further studied. As reported earlier^{3/}, BSA with adjuvant stimulated much higher (> 3 times) and more sustained levels of antibody than soluble or alum-precipitated BSA during the 30-60 day period following immunization. Arbitrarily chosen samples were compared in antigen binding and precipitin tests. Binding capacity was much greater than precipitating activity in early bleedings. As synthesis of precipitating antibody approached a maximum at 21-60 days, total binding and precipitin values were almost equal.

IgG and IgM fractions (obtained by gel filtration) of these sera are being studied to determine if the larger binding capacity in early bleedings reflects the presence of poorly precipitating IgM antibody in the sera.

One year after a primary series of immunization of rabbits, no antibody activity could be detected in their sera. All groups were then given 10 mg of soluble BSA, and a prompt anamnestic response occurred. Those animals originally immunized with BSA in adjuvant produced antibody levels only slightly higher than those following the first series. The animals immunized with soluble or alum-precipitated BSA produced ≥ 3 times the amount measured following the first series. After the booster, the sera of

all 3 groups contained about the same amounts of antibody. Binding and precipitin determinations of each serum showed little difference, indicating a typical IgG anamnestic response. Four weeks after the first booster dose, a second dose of 10 mg BSA was given to each animal, and they were then bled out 12 days later.

The differences observed in responses to a first and second immunization suggest that, following the first series, an antigen with adjuvant both primes and triggers antibody synthesis at nearly maximum levels. A year later, the priming is still present. Soluble or alum-precipitated antigen in the first series seems to be able to prime effectively, but lacks the ability to trigger and maintain high levels of antibody synthesis. The magnitude of the immune status of the animal can only be shown later by the boosters which trigger the large primed cell population into antibody synthesis. Therefore, in 30-60-day experiments in animals immunized with soluble or alum-precipitated antigen, serological levels of antibody need not reflect the true immune response of the animals.

In addition to measurements of the capacity of antibodies to bind antigen, certain properties of these antigen-antibody combinations, which contribute to the ultimate fate of antigen in the host, particularly, biologically active toxins and living microorganisms, are of obvious importance in determining the protective efficacy of antibodies stimulated by various vaccine preparations and regimens, as well as that of therapeutic antiserum.

It has been reported recently^{4,5/} that, when peptide fragments of the immunoglobulins were prepared by enzyme hydrolysis of preformed antigen/antibody (Ag/Ab) precipitates, Ag in the complex seemed unaffected by the protease employed. This observation is diametrically opposed to the usually accepted view that antibody exerts its protective effect by aiding degradation of antigens. It was, therefore, of interest to explore this question further, using unfractionated antiserum and with varying relative concentrations of antigen and antibody to examine soluble complexes as well as preformed precipitates.

In these studies, varying amounts of ^{131}I -labelled BSA were added to a constant volume of whole rabbit antiserum. Pronase was added, and, after a suitable period of digestion, the trichloroacetic acid soluble label was counted as a measurement of ^{131}I -BSA digested.

The presence of BSA antibody provided significant protection against enzyme attack in all cases. Inhibition of enzyme hydrolysis of BSA varied from 35-95% in different antisera; inhibitory activity was roughly proportional to antibody content, as measured by binding capacity. The phenomenon occurred over a wide range of antigen concentrations, from antibody excess to antigen excess. Preincubation of BSA in antisera was unnecessary. Apparently, the initial, instantaneous binding of antigen to antibody was sufficiently rapid to block enzyme action on the antigen.

Immunoglobulin class, tissue enzymes, and nature of antigen may play roles in this observation and should be explored; such studies are planned.

Summary:

In early bleedings of groups of rabbits hyperimmunized with BSA in different physical states, there was more binding than precipitating activity. But in later, and in booster bleedings, measurements of the binding capacity and precipitins were similar. Following a primary series of immunization, BSA in Freund's adjuvant produced higher, more sustained levels of antibody than soluble or alum-precipitated BSA. As evidenced by response to boosters a year later, all methods produced about the same levels of "priming."

Combination of BSA antibodies with "specific" antigen in preformed aggregates or in whole serum significantly blocks the degradation of BSA by pronase.

Publications:

None.

LITERATURE CITED

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3. U. S. Army Medical Unit. 1 July 1968. Annual Progress Report, FY 1968, p. 125 to 127. Fort Detrick, Maryland.
4. Cebra, J. J., D. Divol, and E. Katchalski. 1962. Soluble complexes of antigen and antibody fragments. J. Biol. Chem. 237:751-759.
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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 02 102: Development and Evaluation of an Effective Vaccine Against Pneumonic Plague

Reporting Installation: U. S. Army Medical Research Institute of Infectious Diseases
Forest Glen Section
Washington, D. C.

Division: Microbiology

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Authors: John D. Marshall, Jr., LTC, MSC
Charles O. Roberts
Daniel N. Harrison

Reports Control Symbol: RCS-MEDDH-288(R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
				DA OL0833	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8. DISB'N INSTR'N	9. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES:*		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62706A		1B662706A096		02	
b. CONTRIBUTING		62124011		1B622401A096		02	
c. CONTRIBUTING		CDOG 1212b(9)					
11. TITLE (Precede with Security Classification Code)*							
(U) Development and evaluation of an effective vaccine against pneumonic plague							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS*							
003500 Clinical Medicine; 004900 Defense; 003200 CW, BW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
62 12		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (In thousands)	
b. NUMBER: NA				69		4	
c. TYPE:				FISCAL YEAR		CURRENT	
d. AMOUNT:				70		4	
e. KIND OF AWARD:				f. CUM. AMT.		85	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: USA Medical Research Institute of Infectious Diseases				NAME: Microbiology Division			
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RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Crozier, D.				NAME: Marshall, J. D.			
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21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence considered.				NAME: Roberts, C. R.			
				NAME: Harrison, D. N.			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) <u>Pasteurella pestis</u> ; (U) Plague; (U) Pneumonic plague vaccine; (U) Antigens; (U) Immunization							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) Determine the factors influencing the susceptibility to plague infection and the most appropriate method to prevent the infection.							
24 (U) Using standard methods, strains of <u>Pasteurella pestis</u> are tested for their physiological and virulence characteristics. Serological tests are performed on wild and laboratory animals for the detection of anti- <u>P. pestis</u> Fraction I antibody.							
25 (U) 68 07 - 69 06 - Antimalarial chemoprophylactic drugs in concentrations up to 10 times normal doses did not protect mice from experimental plague infection. All except 4 strains of <u>Pasteurella pestis</u> isolated in the Republic of Vietnam and tested were similar in respect to physiological and virulence characteristics. The 4 avirulent strains were negative for the Pesticin I, fibrinolysin, coagulase complex. A Fraction I inhibition test was developed to differentiate between true antiplague titers and nonspecific heterophile-like reactions. Fitzsimons Army Hospital and Rocky Mountain Arsenal were not involved in the plague epizootic encountered in the city of Denver, Colorado in the summer of 1968.							

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 02 102: Development and Evaluation of an Effective Vaccine Against Pneumonic Plague

Description:

Determine the factors influencing the susceptibility to plague infection and the most appropriate method to prevent the infection.

Progress, Part I:

A series of experiments were conducted to determine the effect of malaria chemoprophylaxis on the course of experimental plague in mice. The following drugs and drug combinations were administered by stomach tube to groups of 30 mice each:

Chloroquin (C) weekly
Primaquin (P) weekly
Paramethimine (Para) weekly
C + P weekly
Di-amino Di-phenyl Sulfone (DDS) daily
DDS daily and C + P weekly
DDS weekly and Para weekly

Dosages for each treatment regime were calculated so that groups of animals received 0.1, 0.5, 1.0, 5.0, and 10.0 X the human doses on a weight basis. All animals were pretreated for 2 weeks. Ten animals from each group were bled to determine blood levels of therapeutic agents. The 20 remaining mice in each group and 20 control mice were inoculated subcutaneously with 200 LD₅₀ of Pasteurella pestis strain 195/P. All animals died during the 10-day test period.

The average time to death was 4.5-5.1 days, except for the 10 X DDS groups where it was 7.9-8.3 days.

Summary, Part I:

Antimalarial chemoprophylactic drugs in concentrations up to 10 X normal doses did not protect mice from experimental plague infection.

Progress, Part II:

During the reporting period, a series of 700 cultures of P. pestis isolated in Vietnam during the period 1963-1968 were characterized for physiological activity according to the criteria of Baltazard et al.^{1/} and for virulence factors by the methods of Brubaker and Surgalla,^{2/} Higuchi and Smith,^{3/} and Beesley et al.^{4/} Strains isolated from the following human clinical disease forms were tested: bubonic, septicemic, primary, and secondary pneumonic, and asymptomatic pharyngeal plague. In addition, strains obtained from domestic, para-domestic, sylvatic animals, and their fleas were included. Geographic distribution of the isolates encompassed the entire country. The strains had been isolated throughout the epidemics so that there were representatives of the onset, peak and decline of no less than 10 well-defined outbreaks.

The most striking observation was the homology of the strains. No significant differences were detected between strains from different geographic location, time of isolation during an epidemic, species from which isolated or clinical severity of the case yielding the isolate. The following are the characteristics of the Vietnam isolates: acid without gas from dextrose and maltose; no acid or gas from lactose, sucrose, glycerol, rhamnose, melibiose; nitrates reduced to nitrate; indole not formed; hydrogen sulfate not produced; urease not produced; Fraction I produced; calcium-dependent; pigmented on heme and Congo Red agar; Pesticin I, II, fibrinolysin, coagulase positive; and virulent for white mice. Four strains, 0.57%, were found to be Pesticin I, fibrinolysin, and coagulase negative. These strains were isolated from 4 separate villages over a period of 8 months. All were from bubo aspirates, with the exception of 8 strains previously reported, moderately resistant to streptomycin. All strains were sensitive to 2 µg of streptomycin, chloramphenicol, tetracycline, oxytetracycline, chlortetracycline, neomycin, and colimycin. Sulfa resistance was directly correlated with inoculum concentration. When < 400 organisms were inoculated, strains were sensitive to 2 mg % sulfa. When the inoculum contained > 400 organisms, the majority of strains were resistant to 20 mg %.

Summary, Part II:

All except 4 strains of P. pestis isolated in the Republic of Vietnam and tested were similar with respect to physiological and virulence characteristics. The 4 avirulent strains were negative for the Pesticin I, fibrinolysin, coagulase complex.

Progress, Part III:

During serological studies of wild and laboratory animals for P. pestis Fraction I antibody, the necessity for an inhibition control became apparent. Heterophile-like antibodies were found in 30% of the wild rodent sera tested. The inclusion of an inhibition control in which each serum was diluted with buffered saline containing 25 µg/ml of Fraction I permitted the differentiation between the true antiplague titers and the nonspecific heterophile

titer. When a series of guinea pigs immunized with various vaccines including an attenuated plague vaccine was challenged, all animals demonstrating antiplague titers survived challenge with 100 LD₅₀ P. pestis 195/P.

Summary, Part III:

A Fraction I inhibition test was developed to differentiate between true antiplague titers and nonspecific heterophile-like reactions.

Progress, Part IV:

During a plague epizootic in the city of Denver, Colorado, a survey of the sylvatic and para-domestic animals residing in Fitzsimons Army Hospital and Rocky Mountain Arsenal was conducted to determine whether any of these animals were involved. During a 3-week period, 511 animals representing 11 species were trapped or shot. These animals harbored 1,403 fleas of 8 species. Sera were obtained from 254 animals. All animals were necropsied and tissues for culture and animal inoculation were taken. Tissues were pooled by species and capture location. Fleas were pooled by species, host species, and capture location. All tissue and flea pools were cultured and inoculated into susceptible laboratory rodents. Sera were tested for the presence of P. pestis Fraction I antibody. All tests were negative indicating that at the time of survey, both Fitzsimons Army Hospital and Rocky Mountain Arsenal were free of plague and had been free for a period of at least 1 yr prior to the recognized beginning of the Denver epizootic.

Summary, Part IV:

Fitzsimons Army Hospital and Rocky Mountain Arsenal were not involved in the plague epizootic encountered in the city of Denver, Colorado in the summer of 1968.

Installation of aerosol exposure equipment has been completed.

Presentations:

1. Marshall, J.D., Jr., Immunofluorescence studies in fungal infections. Presented at the Veterans Administration Hospital Reference Laboratory, Washington, D.C., 2 May 1968.
2. Marshall, J.D., Jr., Plague. Presented at the Armed Forces Institute of Pathology, Postgraduate Course on Special Environmental Pathology-Southeast Asia, Washington, D.C., 22 May 1968.
3. Marshall, J.D., Jr., Plague. Presented at the Global Medicine Course, Walter Reed Army Institute of Research, 5 July 1968, 26 February 1969, and 8 July 1969.

4. Marshall, J.D., Jr., Donald H. Hunter, Llewellyn J. Legters, Nhu-Tran-Quy, and D.C. Cavanaugh, Survey of respiratory infection among persons exposed to Pasteurella pestis: The gradation of clinical symptoms. Presented at the Eighth International Congress on Tropical Medicine and Malaria, Teheran, Iran, 7-15 September 1968.

5. Marshall, J.D., Jr., Plague and tularemia. Presented at the Department of Microbiology Guest Lecturers Program, Howard University, Washington, D.C., 24 March 1969.

Publications:

1. Cavanaugh, D.C., H.G. Dangerfield, D.H. Hunter, R.J.T. Joy, J.D. Marshall, Jr., D.V. Quy, S. Vivona, and P. Winters. 1968. Some observations on the current plague outbreak in the Republic of Vietnam, *Amer. J. Pub. Health* 58:742-749.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 300: Immunologic Studies With The Spotted Fever Group of Rickettsiae

Reporting Installation: U.S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

Division: Virology

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Authors: Marie L. Miesse, B.S.
Emerson L. Shroyer, Major, VC

Reports Control Symbol: RCS-MEDDH-288(R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a DA OL0885	2. DATE OF SUMMARY ^a 69 07 01	REPORT CONTROL SYMBOL DD-R&E (AR) 636	
3. DATE PREV SUMRY 68 07 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY ^a U	6. WORK SECURITY ^a U	7. REGRADING ^a NA	8. DES'N INSTR ^a DE	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	9. LEVEL OF SUM A. WORK UNIT
10. NO./CODES: ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62706A	1B662706A096	02	300			
b. Contracted	62124011	1B622401A096	02				
c. Contracted	CDOG 1212b(9)						
11. TITLE (Precede with Security Classification Code) ^a (U) Immunologic studies with the spotted fever group of rickettsiae							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a 003500 Clinical Medicine; 004900 Defense; 003200 CW, BW, RW							
13. START DATE 67 07		14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. FUNDS (in thousands)	
a. DATES/EFFECTIVE:		EXPIRATION:		PRECEDING		2. PROFESSIONAL MAN YRS	
b. NUMBER: ^a				69		2	
c. TYPE: NA		d. AMOUNT:		CURRENT		8	
e. KIND OF AWARD:		f. CUM. AMT.		70		2	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a USA Medical Research Institute of Infectious Diseases ADDRESS: ^a Fort Detrick, Md 21701				NAME: ^a Virology Division USA Medical Research Institute of Infectious Diseases Fort Detrick, Md 21701			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Crozier, D.				NAME: ^a Miesse, M. L.			
TELEPHONE: 301 663-4111 Ext 5233				TELEPHONE: 301 663-4111 Ext 4203			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence considered.				NAME: Shroyer, E. L.			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Immunology; (U) Rickettsial diseases; (U) Spotted fevers; (U) Vaccines							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23. (U) Develop a method for immunoprophylaxis against the spotted fever group of rickettsiae.							
24. (U) Propagate representative strains in tissue culture systems. Assess the feasibility of producing rickettsial suspensions of quality and quantity suitable for vaccines for human use.							
25. (U) 68 07 - 69 06 - Representative strains of all group members have been propagated in 3 types of tissue culture. Trial antigens have been prepared with <u>Rickettsia conori</u> , <u>Rickettsia siberica</u> and <u>Rickettsia rickettsi</u> which on microscopic examination compare favorably with conventional yolk sac preparations.							

^aAvailable to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 300: Immunologic Studies With The Spotted Fever Group of Rickettsiae

Description:

Develop a method for immunoprophylaxis against the spotted fever group of organisms.

Progress:

Seed Materials. Studies dealing with the method of production and effect of storage have continued. Several preparations of both crude and washed suspensions were prepared from infected yolk sacs.

The crude 20% preparations were prepared by adding 4-ml volumes of Snyder I¹/ diluent (SPG) per gram of drained stripped yolk sacs and emulsifying this mixture in a blender. After centrifugation at 150 X g for 10 min the midzone between sediment and fat was collected, dispensed in small samples, and stored at -60 C.

Washed suspensions were prepared by differential centrifugation of crude suspensions. Sediments from $\geq 6,000$ g centrifugation were resuspended in SPG and centrifuged at 150 X g to remove cellular debris. Alternate high and low speed centrifugation was repeated for 3 cycles. The final low speed supernatant was collected, dispensed, and stored in the freezer.

A few days after storage, samples were titrated in embryonated eggs and thereafter were used in egg, guinea pig and tissue culture studies. It was anticipated that the washed rickettsiae would cause more uniform infections than the crude preparations. However, comparative titrations of crude and washed Rickettsia rickettsi and R. australis in guinea pigs or eggs failed to establish significant differences in infectivity patterns.

After a period of 6 months one of the freezers utilized for storage of the materials failed. Re-titration of preparations in this freezer revealed a considerable loss of titer. Work is in progress to re-establish stocks of high titered seeds.

Tissue Culture. Passage studies. Representative strains of the subgroups have been propagated in tissue culture. Each was passaged in

MK₂, Vero and chick embryo cell cultures. With each of the cell lines employed, a cytopathic effect (CPE) was obtained.

Comparison of a single strain in several cell lines revealed different patterns of CPE, although changes occurred at about the same time. This variation in CPE is probably due to inherent structural differences of the cell monolayers. Stained smears of passage material did not show significant differences of rickettsial yields with the cell lines employed.

Conversely when several strains were propagated in cultures of one cell type, differences in time of CPE and yield were noted when comparable quantities of inocula were employed.

The onset of CPE was first evident with the appearance of small patches of necrotic cells. This was followed by a spreading infection until the entire monolayer was affected. Most of the infected cells remained attached to the walls of the culture flask. Serial passages were made by disrupting the infected cultures with sterile glass beads. The resulting crude material which contained supernatant medium, rickettsiae and cell debris was used for the next passage.

Table I summarizes tissue culture passages made with various members of the group.

TABLE I. PASSAGE OF VARIOUS MEMBERS OF THE SPOTTED FEVER GROUP IN TISSUE CULTURES

RICKETTSIA (strain)	NUMBER SERIAL CELL PASSAGES		
	MK ₂	CE	VERO
<u>R. conori</u> (boutonneuse)	15	5	3
<u>R. siberica</u>	7	5	3
<u>R. australis</u>	10	5	1
<u>R. rickettsi</u> (S. Smith)	11	5	3
<u>R. rickettsi</u> (Bitterroot)	3	3	1
<u>R. akari</u>	2	ND	1

These studies established that representative strains could be grown in a variety of cell types. However, erratic results were frequently encountered. Day of onset of CPE was variable, and passage failures occurred.

Studies have been initiated to determine possible causes for the variable results in an attempt to establish whether each strain requires a particular

set of conditions for propagation.

Storage. Passage studies revealed a significant loss of viability when material was stored in the freezer. A study was made to select a stabilizing storage medium for a tissue culture harvest of R. siberica.

The culture was sedimented and resuspended by aspiration through a 25 gauge needle in a 50% volume of medium 199. Dilutions (1:10) of this suspension were made in: 1) medium 199; 2) medium 199 + 0.5% human serum albumin (HSA); 3) SPG; and 4) SPG + 0.5% HSA. Each 1:10 dilution was divided in 4 samples and stored at -60 C. Titrations were performed at intervals in embryonated eggs and tissue cultures. Table II gives results of the egg titrations. Values for median egg lethal doses (ELD₅₀) varied from one storage period to the next. However, in all 3 series, cultures in SPG medium had a higher titer than those stored in medium 199. Addition of HSA did not result in higher titers in either medium. Titrations were also accomplished in cell cultures, but were inconclusive because of deterioration of the monolayers at endpoint dilutions prior to CPE. Daily microscopic readings showed more rapid CPE when cells had been inoculated with low dilutions of rickettsiae stored in HSA, whether medium 199 or SPG was used. Thus apparent contradiction with ELD₅₀ results may be due, not to stability on storage, but to enhancement of cell penetration by HSA. Cohn, et al^{2/} reported that the penetration index of suspended tissue culture cells was increased when the organisms were suspended in bovine serum albumin. Studies will be made to determine effect of HSA on monolayer yields.

TABLE II. TITERS OF R. SIBERICA AFTER STORAGE AT 60 C.

STORAGE MEDIUM	ELD ₅₀		
	73 days	86 days	100 days
199	2.5	2.6	3.0
199-HSA	2.9	2.5	3.0
SPG	3.0	4.5	4.5
SPG-HSA	3.5	4.3	4.5

Glutamine. Reports of Cohn et al^{2/} that the presence of glutamine increased the penetration index of R. tsutsugamushi led to experiments to determine if glutamine would increase yields of R. conori when added to chick embryo monolayers just prior to the adsorption period. Three attempts failed to demonstrate growth differences between glutamine-treated or untreated cultures. Onset and intensity of CPE were the same for both cultures in every case. Sub-cultures of 2- 3- and 5-day harvests of glutamine-treated cells produced CPE at the same rate as untreated control cultures. Penetration studies were not done. It was concluded that

JOB, DPG

addition of glutamine to the cultures during the penetration period did not enhance the growth of R. conori.

Assay systems. A variety of methods has been employed to achieve a dependable assay system in tissue culture. CPE titrations and plaque titrations can be employed. However, both are dependent on healthy long-lived cells, since CPE and plaquing occur much later in endpoint dilutions than in low dilutions. Once the monolayer is infected, it is necessary to maintain the cells in a healthy condition while minimizing cell multiplication.

In attempts to develop a reproducible plaque system with R. conori, 7 of 12 experiments failed because the agarose-overlaid cells did not survive the 6-day time period established for plaque assays.

When titrations for CPE were done with liquid media, problems of overgrowth were encountered.

A titration series of R. conori was made in quadruplicate in chick embryo cells. Two of the sets were fed daily with medium 199 + 5% calf serum and 2 received medium 199 only. Monolayers given medium 199 only could be observed for CPE through day 14, while those which received the enriched medium sloughed off the flasks' walls by day 6. Although CPE was present in the lowest dilution (10^{-1}) by day 3, it became visible at the endpoint dilution (10^{-6}) on day 11.

The problem of maintaining the proper metabolic balance is complex, since many interdependent factors are involved, such as cell type and method of preparation, nutritional requirements, and temperature of incubation. Although these problems have plagued attempts to establish reliable assay systems, they have not been a major concern in production of rickettsial antigens in tissue culture.

Antigens. The boutonneuse fever strain of R. conori (BF) has been used as a model in production of experimental tissue culture antigens. This strain was selected because previous observations have indicated relatively rapid and prolific growth from high titer inocula in chick yolk sac and a variety of tissue culture cells. The primary chick embryo monolayer was selected because it will support rickettsial growth and is considered less apt to be oncogenic than established cell lines. Either roller bottle or stationary cultures have produced satisfactory results. It has been possible to prepare rickettsial suspensions of relative purity in these systems with a minimal amount of processing.

Stained smears studied under oil immersion reveal numerous intact rickettsiae with very little cell debris. These smears compare very favorably with those from processed yolk sac preparations, which are usually sparse in organisms and rich in debris after elaborate production and purification procedures. Assays for antigenicity and immunogenicity have been delayed until the laboratory moves to new quarters.

Summary:

All members of the spotted fever group of rickettsia have been propagated in MK₂, Vero and chick embryo cells. Trial antigens have been prepared from tissue cultures.

Publications:

None.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 402: Comparative Studies of Various Routes of Immunization with Arbovirus Vaccines

Reporting Installation: U. S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

Division: Animal Assessment

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Authors: Ralph W. Kuehne, B.S.
Clyde W. Boyd

Reports Control Symbol: RCS-MEDDH-288(R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OL0836	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMRY ^a	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DISEN INSTR ^a	9. SPECIFIC DATA- CONTRACTOR ACCESS	
68 07 01	D. CHANGE	U	U	DA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO A. WORK UNIT	
10. NO./CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62706A		1B662706A096		02	
b. CONTINGENT		62124011		1B622401A096		02	
c. CONTINGENT		CDOG 1212b(9)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) Comparative studies of various route of immunization with arbovirus vaccines							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
003500 Clinical Medicine; 004900 Defense; 003200 CW, BW, RW							
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61 08		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PREVIOUS		b. FUNDS (in thousands)	
b. NUMBER: ^a				FISCAL		69	
c. TYPE:				YEAR		2	
d. KIND OF AWARD:				CURRENT		10	
e. AMOUNT:				70		2	
f. CUM. AMT.						10	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME ^a USA Medical Research Institute of Infectious Diseases				NAME ^a Animal Assessment Division			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
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				NAME: Boyd, C. W.			
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(U) Encephalitis, equine (EEE, VEE, WEE); (U) Arboviruses; (U) Vaccines; (U) Immunization							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Evaluate routes of and dosages for immunization of susceptible hosts with arbovirus vaccines.							
24. (U) Laboratory animals will be immunized by varying routes and dosages and challenged at appropriate times subsequently.							
25. (U) 68 07 - 69 06 - The protective effect of various vaccine combinations: TC-83 strain Venezuelan equine encephalitis (VEE), Clone 15 strain Western equine encephalitis (AWEE), small plaque mutant (SPM) of Eastern equine encephalitis (EEE) and inactivated EEE (IEEE) was examined in guinea pigs. TC-83 VEE and AWEE strains gave complete homologous protection, but the IEEI vaccine gave only partial homologous protection and the SPM-EEE only negligible protection. IEEI and TC-83 VEE gave a low-level, reciprocal cross-protection. TC-83, combined with either of the EEE vaccines, greatly enhanced the protection afforded against EEE. Similar enhancement was seen against EEE when AWEE was added to either IEEI or TC-83. The administration of a single dose of trivalent or tetravalent vaccine which included TC-83, AWEE and IEEI afforded excellent protection against all 3 virulent virus challenges without evidence of interference.							

^aAvailable to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Warfare (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 402: Comparative Studies of Various Routes of Immunization with Arbovirus Vaccines

Description:

Evaluate routes of dosages for immunization of susceptible hosts with arbovirus vaccines.

Progress:

Emphasis during this period was on determination of the effectiveness of combining previously studied arbovirus vaccines, administering combinations parenterally to guinea pigs and looking for interference and protection against homologous and heterologous challenge. Maurer *et al*¹ used a tri-valent vaccine successfully in guinea pigs in 1952 consisting of the 3 killed vaccines against Venezuelan, Eastern, and Western equine encephalitis (VEE, EEE, WEE), but no information has been reported incorporating attenuated vaccines.

A 0.5 ml inoculum containing 5,000 intraperitoneal (IP) median immunizing doses (ID₅₀) of attenuated VEE (strain TC-83) and Clone-15 WEE (AWEE) and <1 guinea pig (GP) IPID₅₀ of inactivated Eastern equine encephalitis (IEEE) and/or small plaque mutant EEE (SPM-EEE) were used in all combinations of di-, tri- and tetravalent vaccines. One thousand LD₅₀ of Trinidad strain VEE by IP inoculation and B-11 strain WEE and Texas strain EEE by intracerebral (IC) inoculation were used 21 or 27 days postvaccination to test the *in vivo* protection afforded the guinea pigs by the various vaccines administered. All controls at each challenge level died, and where differences due to challenge time occurred in vaccinated animals, it is shown in Table I.

The effectiveness of various mono- and divalent combinations against challenge with VEE and WEE is shown in Table I. The vaccines were administered by both the subcutaneous (SC) and IP routes, but since little difference was noted in protection, results of both routes have been combined.

Virtually complete homologous protection exists whenever VEE or WEE vaccine is present (the 96% protection obtained when WEE and SPM-EEE are combined represents only 1 animal in a group of 24). IEEI alone or combined

with WEE afforded some protection against VEE challenge, whereas SPM-EEE gave protection only against VEE combined with WEE. IEEE combined with VEE resulted in 17% protection against WEE challenge (Cole^{2/} obtained 40% protection with this combination in hamsters, but with a more potent IEEE vaccine than the one used in these studies).

TABLE I. SURVIVAL OF GUINEA PIGS AFTER CHALLENGE WITH 10^3 LD₅₀ VEE OR WEE VIRUS.

VACCINE	% SURVIVAL AFTER CHALLENGE WITH	
	VEE	WEE
None	0	0
IEEE	10	0
SPM-EEE	0	0
AWEE	ND	100
IEEE + AWEE	11	100
SPM-EEE + AWEE	17	96
TC-83 VEE	100	0
IEEE + TC-83 VEE	100	17
SPM-EEE + TC-83 VEE	ND	4
AWEE + TC-83 VEE	100	100

The effectiveness of these same combinations of vaccines against challenge with virulent EEE 21 and 27 days postvaccination is shown in Table II. No significant difference is seen between SC and IP routes (shown in parentheses) of vaccine administration.

The SPM-EEE vaccine produces no better protection against EEE challenge than does the TC-83 vaccine; however, the IEEE vaccine produces significantly greater protection against EEE challenge than does any other monovalent vaccine. That attenuated VEE does give cross-protection against EEE has been noted by Allen,^{3/} Hearn,^{4/} and Cole.^{5/} Significantly greater protective effect was seen with IEEE when protection was measured 27 days postvaccination, rather than at 21 days. Because of this lesser protection seen at 21-day vs. 27-day challenge with IEEE alone, an increased protection could be seen when

combinations of IEE and SPM-EEE, WEE or VEE were used and challenge was given on day 21; however, this difference was not seen with any other combination.

TABLE II. SURVIVAL OF GUINEA PIGS AFTER IC CHALLENGE WITH 10^3 LD₅₀ TEXAS STRAIN EEE VIRUS.

VACCINE	% SURVIVORS	
	21 Day Challenge	27 Day Challenge
IEE	38 (35, 40) ^{a/}	67 (65, 71)
SPM-EEE	5 (0, 9)	7 (7, 8)
AWEE	1 (1, 0)	4 (8, 0)
IEE + AWEE	67 (63, 69)	71 (67, 78)
SPM-EEE + AWEE	8 (ND, 8)	0 (ND, 0)
TC-83 VEE	19 (17, 20)	21 (17, 25)
IEE + TC-83 VEE	77 (83, 75)	89 (100, 83)
SPM-EEE + TC-83 VEE	46 (50, 42)	67 (50, 83)
AWEE + TC-83 VEE	43 (41, 46)	23 (29, 17)
IEE + SPM-EEE	75 (ND, 75)	83 (ND, 83)

a. (SC, IP)

Attenuated VEE and attenuated WEE have an adjuvant or synergistic effect on EEE protection when added to IEE vaccine, or when added to each other. In fact, VEE combined with IEE affords as good protection as the combination of the 2 EEE vaccines. TC-83 VEE also showed an adjuvant effect when combined with SPM-EEE on both the 21-day and 27-day challenge, but WEE did not show this effect.

Dr. Cole, Virology Division, USAMRIID, states that there is good protection in hamsters with both SPM-EEE and his IEE vaccine, which is more potent than the inactivated EEE vaccine used in these studies (obtained from Walter Reed Army Institute of Research); he did not observe any adjuvant effect of TC-83 VEE vaccine. However, he noticed an adjuvant effect of attenuated VEE when added to a poorer IEE vaccine in terms of resistance to EEE

challenge. It may be that the effect is masked when a vaccine-host system is used, where good protection is afforded by the EEE vaccine alone.

Protection data afforded by trivalent combinations is presented in Table III. Trivalent vaccine with the Walter Reed IEE or SPM-EEE gives good protection against all 3 viruses when measured at 27 days postvaccination, especially when IEE is used. When a single dose of trivalent vaccine with this inactivated vaccine is used, followed by a booster of IEE at 28 days, good protection is achieved, but no better than that seen without a booster inoculum. However, when trivalent vaccine with SPM-EEE is used, followed by a 28-day booster of SPM-EEE, solid protection is achieved by day 21 postvaccination. Two doses of SPM-EEE alone given 28 days apart results in only 67% survival of an EEE challenge at day 27, so the addition of the VEE and WEE vaccines greatly enhances protection against EEE. No interference is seen with the trivalent combinations. Dr. Cole found a reduction in protection against WEE with a trivalent attenuated vaccine in hamsters, but this is not observed in our series.

TABLE III. RESPONSE OF GUINEA PIGS GIVEN TRI- AND TETRAVALENT VACCINES TO CHALLENGE WITH 10^3 LD₅₀ VEE, WEE AND EEE VIRUSES.

VACCINE	% PROTECTION AFTER CHALLENGE WITH			
	VEE	WEE	EEE	
			21 Days	27 Days
IWEE + AWEE + TC-83 VEE	100	96	75	95
SPM-EEE + AWEE + TC-83 VEE	96	96	71	83
IEE + AWEE + TC-83 VEE + 28-day IEE booster	ND	ND	ND	91 ^a /
SPM-EEE + AWEE + TC-83 VEE + 28-day SPM-EEE booster	ND	ND	100	ND
IEE + SPM-EEE + AWEE + TC-83 VEE	100	100	92	ND

a. With 8% fatal anaphylaxis

Also shown in Table III is the effect of a tetravalent vaccine (TC-83 VEE, AWEE, IEE and SPM-EEE). A single dose administered by the IP route results in good protection at day 21 against challenge with EEE.

In man, 2 or 3 doses of IEE are required to produce a good log neutralization index. These results suggest that the same index might be attained

after a single dose, if attenuated VEE vaccine were added to the IEE. The data further suggest that it may be advantageous to give VEE, WEE and EEE vaccines simultaneously. In addition to reducing the number of inoculations and the immunization time now required, earlier and as-good-or-better protection might result against all 3 viruses.

Summary:

Data are presented showing the effectiveness of various di-, tri- and tetravalent arbovirus vaccines administered parenterally to guinea pigs. Attenuated VEE vaccine added to either of the inactivated or attenuated EEE vaccines used increases protection against EEE challenge. A single dose of trivalent or tetravalent vaccine, consisting of TC-83 VEE, attenuated WEE and IEE and/or attenuated EEE virus, results in good protection against challenge with all 3 virulent counterparts without evidence of interference. In addition, earlier and better protection against EEE challenge occurs over that seen when these low-titered EEE vaccines are used alone.

Publications:

None.

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5. U. S. Army Medical Unit. 1 July 1968. Annual Progress Report, FY 1968. p. 153 to 161. Fort Detrick, Maryland.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 403: Cross-Immunity Within the A Group of Arboviruses

Report Installation: U. S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

Division: Virology

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Authors: Francis E. Cole, Jr., Ph.D.
Helen T. Hargett

Reports Control Symbol: RCS-MEDDH-288(R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
				DA OL0837	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8. DISSEM INSTRN	9. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER		WORK UNIT NUMBER		
a. PRIMARY	62706A	1B662706A096	02		403		
b. CONTINUITY	62124011	1B622401A096	02				
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11. TITLE (Precede with Security Classification Code)*							
(U) Cross-immunity within the A group of arboviruses							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS*							
003500 Clinical Medicine; 004900 Defense; 003200 BW, CW, RW							
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17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
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b. NUMBER: NA				FISCAL YEAR		30	
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19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: USA Medical Research Institute of Infectious Diseases				NAME: Virology Division			
ADDRESS: Fort Detrick, Md 21701				ADDRESS: USA Medical Research Institute of Infectious Diseases			
				Fort Detrick, Md 21701			
				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
RESPONSIBLE INDIVIDUAL				NAME: Cole, Jr., F. E.			
NAME: Crozier, D.				TELEPHONE: 301 663-4111 Ext 4203			
TELEPHONE: 301 663-4111 Ext 5233				SOCIAL SECURITY ACCOUNT NUMBER:			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence considered.				NAME: Hargett, H. T.			
				NAME: DA			
22. (U) Vaccines; (U) Arboviruses; (U) Immunization; (U) Encephalitis, equine (VEE, EEE, WEE)							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Evaluate experimental attenuated and inactivated group A arbovirus vaccines for their ability to induce protection against other members of the group.							
24. (U) Adult hamsters are inoculated with group A arbovirus vaccines in appropriate combinations and sequences. Response is determined by challenge with virulent strains and by serological techniques.							
25. (U) 68 07 - 69 06 - Immunization of hamsters with formalin-inactivated VEE vaccine (125 or 2000 median immunizing doses) failed to provide any protection against challenge with virulent EEE and WEE viruses, although homologous protection was absolute. This supports the view that the cross-protection seen in hamsters immunized with attenuated VEE is due only in small part to antigens common to the 3 viruses.							
Studies in hamsters with attenuated strains of VEE, EEE and WEE viruses showed that: (1) administered singly they provide excellent homologous protection; (2) sequence of administration had a marked effect only on the degree of VEE resistance in animals given EEE and WEE virus strains viz., WEE followed by EEE induced only 23% protection, whereas the reverse sequence protected 68% of the hamsters; (3) homologous protection by WEE virus is depressed when the virus is administered simultaneously with VEE and/or EEE virus; (4) simultaneous administration of VEE and EEE depresses homologous protection by EEE; (5) all 3 attenuated viruses produced some deaths, alone or in combination e.g. administered singly VEE killed 10-20% of the hamsters; WEE, 3-5%; and EEE, 15-30%; the WEE-EEE mixture killed 30-35%, while all other combinations had an average death rate of 9.7%.							

* Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 403: Cross Immunity Within the A Group of Arboviruses

Description:

Evaluate experimental attenuated and inactivated group A arbovirus vaccines for their ability to induce protection against other members of the group.

Progress:

Previous studies^{1,2,3/} employing young adult Golden Syrian hamsters have provided data to support the following generalizations: (1) attenuated Venezuelan equine encephalitis (VEE) vaccine rapidly induces a state of protection against challenge with VEE, eastern and western equine encephalitis (EEE, WEE) viruses which is virtually complete throughout the period of vaccine viremia; (2) protection against EEE and WEE viruses persists after vaccine viremia abates, with 59 and 37% protection, respectively, occurring as late as day 60 postvaccination (the longest period evaluated); (3) attenuated VEE vaccine does not increase heterologous protection when administered with inactivated EEE and/or WEE vaccines, regardless of sequence of administration; and (4) attenuated VEE vaccine has no effect on homologous or heterotypic protection when administered with inactivated EEE or WEE vaccines of high potency; however, some enhancement of homologous protection does occur with low potency vaccines.

During the year studies included evaluation of cross-protection induced by inactivated VEE, and attenuated strains of EEE and WEE viruses.

Protection Induced by Formalin-Inactivated VEE Vaccine. Little is known about the mechanism of the well-documented cross-protection induced by attenuated VEE vaccine. An earlier report^{3/} stated that a potent inactivated VEE vaccine failed to induce cross-protection when given in a single, 0.5 ml injection (~125 hamster immunizing doses). It has been postulated that the cross-protection seen in hamsters administered live VEE vaccine may be due only in part to antigens common to EEE, WEE and VEE viruses. Quite apparent are the possibilities that total antigenic mass (presumably lower in inactivated vaccines), exposure of the host to minor antigens (through vaccine virus replication in the host), latent infection of target cells with the vaccine virus,^{4/} or other factors may be involved in the VEE-WEE-EEE cross-protection phenomenon.

To determine the effect of antigenic mass, hamsters were inoculated intraperitoneally (IP) with two 2.5 ml-doses of undiluted, inactivated VEE vaccine (~ 2000 hamster immunizing doses) with a 7-day interval between doses. Twenty-one days after the 2nd dose the animals were challenged IP with $\sim 10^3$ median lethal doses (LD_{50}) of either Cambridge strain EEE virus, California strain WEE virus, or Trinidad strain VEE virus; these strains, dose and route of inoculation were employed for all studies reported herein.

As with the 0.5 ml dose, the two 2.5 ml doses of inactivated VEE vaccine failed to elicit cross-protection. Only 2% (2/100) of the hamsters survived EEE challenge and only 1% (1/100) survived WEE challenge; homologous protection was absolute with 100% (20/20) surviving VEE challenge. Although the antigenic mass administered undoubtedly was much less than that which results from infection with the attenuated VEE vaccine, it is significant that the ~ 16 -fold increase in the quantity of inactivated antigen failed to elicit even a slight degree of cross-protection. Further studies will be conducted on the nature of the VEE-vaccine-induced cross-protection when time and space permit.

Studies with Attenuated Strains of EEE and WEE Viruses. Although potent inactivated EEE and WEE vaccines are currently available for use in man, all studies with these products in animals and man have been short term: ~ 2 months in the case of animals and ~ 1 year with man. It is not possible, therefore, to predict with certainty the persistence of neutralizing antibody (or protection) induced by the inactivated vaccines. In contrast, experience with live vaccines indicate that these may be expected to produce long-lasting immunity. Still other advantages of live vaccines are their ability to induce rapid protection, and generally, a requirement for only one dose. Moreover, numerous investigators^{5,6} have demonstrated that exposure of animals to 2 or more group A viruses can result in a broadened immunological response; significantly, the cross-protection observed by these workers has generally been induced by live virus infections rather than by administration of inactivated virus preparations. Since we have seen no significant cross-protection in hamsters inoculated with various combinations of inactivated VEE, EEE or WEE vaccines, studies were initiated with attenuated strains of EEE and WEE viruses.

A small plaque mutant (SPM) of EEE virus, strain Arth 167 was obtained from Dr. P. H. Coleman, Communicable Disease Center, Atlanta, Georgia, as 13th passage duck embryo tissue culture (DETC) fluid. An additional passage was made in DETC in our laboratory. This material titers $\sim 10^{8.5}$ LD_{50} /ml in suckling mice but is avirulent for the hamster.

Clone 15, B628 strain WEE virus⁷ was obtained from Lederle Laboratories as 2nd passage chick embryo cell (CEC) culture fluid. A further CEC culture passage was made in this laboratory; this material titers $\sim 10^{4.7}$ median immunizing doses (ID_{50} /ml) in hamsters against a WEE challenge.

In separate experiments (Table I) hamsters were inoculated IP with

$\sim 10^3$ ID₅₀ of these attenuated strains and/or attenuated VEE virus using various sequences of administration and all possible combinations. Where sequential schema were employed the separate doses were given 21-30 days apart; challenges were made IP with virulent viruses 21 to 30 days after the last vaccine dose.

TABLE I. RESPONSE TO CHALLENGE IN HAMSTERS VACCINATED WITH ATTENUATED VEE, EEE, AND/OR WEE VIRUSES

LINE NO.	VACCINATION SCHEDULE ^{a/}	% SURVIVORS (NO./TOTAL) AFTER IP CHALLENGE WITH 10 ³ LD ₅₀ OF: ^{b/}		
		WEE	EEE	VEE
1	WEE, VEE	100 (20/20)	82 (45/55)	100 (10/10)
2	VEE, WEE	84 (42/50)	82 (41/50)	100 (13/13)
3	EEE, VEE	32 (16/50)	100 (25/25)	100 (10/10)
4	VEE, EEE	28 (14/50)	94 (47/50)	100 (12/12)
5	WEE, EEE	100 (45/45)	85 (69/81)	23 (63/275)
6	EEE, WEE	96 (24/25)	100 (25/25)	68 (123/180)
7	VEE-WEE-EEE	40 (32/80)	84 (67/80)	98 (59/60)
8	VEE-WEE	70 (21/30)	40 (12/30)	100 (30/30)
9	VEE-EEE	10 (3/30)	73 (23/30)	97 (29/30)
10	WEE-EEE	80 (28/35)	89 (31/35)	11 (11/100)
11	EEE	6 (8/128)	90 (75/83)	7 (5/70)
12	WEE	95 (133/140)	35 (45/130)	1 (1/94)
13	VEE	37 (97/260)	59 (158/268)	98 (190/194)

a. Comma indicates sequential administration of "vaccines;" hyphen indicates simultaneous administration; all "vaccines" administered IP; only 1 dose of a "vaccine" was used.

b. Data are a compilation of results of several experiments.

As shown in Table I the 3 attenuated strains elicited excellent homologous protection when administered singly (lines 11, 12, 13). Of the 3, VEE produced the highest measure of heterologous protection and EEE the lowest.

Sequence of immunization resulted in marked effects in only a few schema. Use of VEE with attenuated WEE (lines 1, 2) or EEE viruses (lines 3, 4) did not result in any significant change in homologous or heterologous protection, with the exception of an increase in protection against EEE challenge in hamsters immunized with VEE and WEE viruses (lines 1, 2); this occurred, however, regardless of sequence. In vaccination schedules employing only attenuated WEE and EEE viruses sequence of administration had no significant effect on homologous protection, but a marked effect on protection against VEE challenge, *viz.*, WEE followed by EEE elicited only 23% protection against VEE (line 5), whereas the reverse order of immunization (line 6) resulted in 68% protection.

Simultaneous administration of the 3 attenuated strains (line 7) resulted in a marked decrease in the protective efficacy of the WEE virus, but had no appreciable effect on the protection induced by EEE or VEE viruses. When administered simultaneously with either VEE virus (line 8) or EEE virus (line 10) the attenuated WEE exhibited a similar depression in its ability to induce homologous protection. The homologous protection induced by EEE virus was depressed when it was administered with VEE virus (line 9), but was unaffected by simultaneous administration of WEE virus (line 10).

Not shown in Table I, but of significance are the death rates of hamsters inoculated with these attenuated strains. Administered singly VEE killed 10-20% of the hamsters, WEE 3-5%, and EEE 15-30%; when administered in combined form the WEE-EEE mixture killed 20-35%, while all other combinations averaged 9.7%, ranging from 6% for the VEE-WEE-EEE mixture to 11% for the VEE-WEE mixture.

The observed death rates may be due to inherent variation in the susceptibility of the hamster to infection with these viruses, genetic recombination of the attenuated strains in the host resulting in a lethal hybrid virus, increased growth and lethal effect of concomitant organisms in the hamster as a result of debility induced by the attenuated strains, or some combination of these factors. Additional, and quite obviously, more sophisticated studies would be required to determine the cause of these "vaccine-induced" deaths.

Evaluation of Combined Attenuated VEE and Inactivated EEE and WEE Vaccines. In preliminary studies on the practical aspects of vaccine administration, hamsters were simultaneously immunized via the IP route with attenuated VEE vaccine, and formalin-inactivated WEE and EEE vaccines. The latter 2 vaccines were prepared and packaged in an identical manner to the vaccines now used in humans. The attenuated VEE vaccine was diluted to contain $\sim 10^3$ hamster ID₅₀/0.5 ml; this material was then used to reconstitute the lyophilized WEE vaccine. The resulting VEE-WEE mixture was then used to reconstitute the lyophilized EEE vaccine. As a result a 0.5 ml hamster dose contained $\sim 10^3$ ID₅₀ of VEE and 10^2 ID₅₀ each of WEE and EEE. Additional animals were given combined WEE and EEE vaccines. All hamsters were challenged 21 days later.

TABLE II. RESPONSE TO CHALLENGE IN HAMSTERS ADMINISTERED COMBINED ATTENUATED VEE AND/OR FORMALIN-INACTIVATED WEE AND EEE VACCINES

VACCINATION SCHEDULE	% SURVIVORS (NO./TOTAL) AFTER CHALLENGE WITH 10^3 LD ₅₀ OF:		
	WEE	EEE	VEE
VEE-WEE-EEE	93 (65/70)	99 (69/70)	100 (10/10)
WEE-EEE	100 (50/50)	100 (50/50)	4 (2/50)
Controls (normals)	0 (0/10)	0 (0/10)	0 (0/10)

These results (Table II) indicate the feasibility of employing reconstituted, attenuated VEE vaccine as diluent for the WEE and EEE vaccine, and moreover, demonstrate that simultaneous administration of the three vaccines results in protection at least as good as that obtained by sequential administration, the current procedure for human vaccinations. The logistical advantages of the new procedure are obvious. Confirmative studies based on immunologic responses in man are planned (see also Work Unit 096 02 002), since suitable vaccines are available for human use.

To date over 50 different immunization schema have been evaluated in hamsters using both attenuated and formalin-inactivated VEE, WEE and EEE viruses. Further cross-protection studies with other members of the A group of arboviruses will require acquisition of additional host-test systems since the hamster is not susceptible to the majority of these viruses. Of greater importance is the need for development and testing of formalin-inactivated vaccines for other members of this virus group (see also Work Unit 096 02 407). It is therefore apparent that further cross-protection studies will be delayed, since personnel limitations require their utilization exclusively for the development, production and testing of the necessary vaccines.

Summary:

Immunization of hamsters with formalin-inactivated VEE vaccine (125 or 2000 ID₅₀) failed to provide any protection against challenge with EEE and WEE viruses, although homologous protection was absolute. This supports the view that the cross-protection seen in hamsters immunized with attenuated VEE is due only in small part to antigens common to the 3 viruses.

Studies with attenuated strains of VEE, EEE and WEE viruses showed that: (1) administered singly they provide excellent homologous protection; (2) sequence of administration had a marked effect only on the degree of VEE resistance in animals given EEE and WEE virus strains viz., WEE followed by EEE induced only 23% protection, whereas the reverse sequence protected

68% of the hamsters; (3) homologous protection by WEE virus is depressed when the virus is administered simultaneously with attenuated VEE and/or EEE viruses; (4) simultaneous administration of VEE and EEE depresses homologous protection EEE; (5) all 3 viruses caused death prior to challenge, e.g. administered singly VEE killed 10-20% of the hamster, WEE 3-5%, and EEE 15-30%; the WEE-EEE mixture killed 20-35%, while all other combinations had an average death rate of 9.7%.

Publications:

None.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 407: Development of Inactivated Group A Arbovirus Vaccines

Reporting Installation: U. S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

Division: Virology

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Authors: Francis E. Cole, Jr., Ph.D.
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23. (U) Produce inactivated group A arbovirus vaccines from selected strains; investigate their capacity to induce serologic response and/or resistance to challenge in test animals.							
24. (U) Arboviruses are propagated in primary cell cultures and inactivated with formalin. Products are tested for safety and potency in animals. Efficacy is determined by subsequent challenge.							
25. (U) 68 07 - 69 06 - Fifteen lots of potent Semliki Forest (SF) virus vaccine have been prepared in roller bottle cultures of chick embryo cells (CEC). The following parameters for SF vaccine production are presented: (1) using a multiplicity of inoculum (MOI) of 0.005 CEC cultures yield maximum titers of virus 18-24 hr postinoculation; (2) up to 300 ml of maintenance medium may be employed without decreasing final potency; and (3) inactivation at 37 C may be carried out with 0.05% formalin for 30-72 hr, or 0.1% formalin for 24-30 hr.							
Utilizing similar methods the following parameters have been established for EEE vaccine production (21 small lots) in CEC cultures; (1) a MOI of ≥ 0.005 ; and (2) high titers of cultures 18-24 hr maintained with up to 300 ml of medium; (3) inactivation with 0.05% formalin is complete in 8-16 hr. Potency tests on EEE vaccines are in progress.							
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BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)
Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties
Work Unit No. 096 02 407: Development of Inactivated Group A Arbovirus Vaccines

Description:

Produce inactivated group A arbovirus vaccines from selected strains of viruses propagated in cell culture; investigate the capacity of these vaccines to induce serological response and/or resistance to challenge in test animals.

Progress:

Previous reports^{1,2/} described a procedure for producing small lots of formalin-inactivated Eastern and Western equine encephalitis (EEE, WEE) vaccines. In brief, this method utilized 3 successive, cumulative passages of infected tissue culture fluids in chick embryo cell (CEC) cultures grown in 250-ml plastic flasks. Each third-passage culture ultimately yielded only 10 ml of virus-containing fluid for further processing into vaccine. Therefore, multiple-handling of large numbers of cultures (~300) was required to produce a single liter of vaccine. As a result this method required many man-hours and necessarily increased the risk of contamination in the final product.

During the past year efforts were directed towards the development of less tedious and time consuming methods for the production of selected group A arbovirus vaccines. Particular emphasis was placed on effective utilization of roller bottle cell cultures, each of which contain the same number of cells as approximately twelve 250-ml plastic flasks.

Virus Strains. Semliki Forest (SF) virus, Sen-1 MB strain was obtained from Dr. W. P. Allen, Fort Detrick as a mouse brain (MB) suspension. The virus was passaged again in suckling mice and a MB suspension was prepared; this material was further passaged once in 9-day-old embryonated eggs, followed by 2 passages in CEC culture. The second passage CEC culture fluid was used as seed virus for vaccine production as well as challenge virus for potency assays.

Eastern equine encephalitis virus, PE-6 strain, was obtained from Walter Reed Army Institute of Research as an egg suspension with a history of 13 intracerebral (IC) mouse passages, followed by 6 passages in embryonated eggs. The virus was subjected to 2 additional passages in

embryonated eggs, with the virus from the second egg passage serving as seed for vaccine production and challenge virus for potency assays.

Virus Titrations. Three-week-old white mice (CD-1 strain of Charles River Mouse Farms, Wilmington, Mass.) were used for all virus titrations. Viruses were diluted in cold phosphate buffered saline (PBS) containing 1% normal rabbit serum. Groups of 5 mice were inoculated IC with 0.03 ml of log₁₀ dilutions of virus-containing fluids and observed for 10 days for deaths. Titration endpoints were determined by the method of Reed and Muench^{3/} and expressed as median lethal doses (LD₅₀) per 1.0 ml.

Preparation of Cell Cultures. Nine-day-old chick embryos were minced and trypsinized according to conventional methods. The resulting cell suspensions were suspended to a final concentration of 4×10^6 cells/ml in a growth medium consisting of Eagle's basal medium containing 10% calf serum and 1% glutamine plus 100 µg/ml each of neomycin, U.S.P. and streptomycin, U.S.P.. Bellco 840 cm² cell production roller vessels (Bellco Glass, Inc., Vineland, N. J.) were seeded with 170-ml portions of the cell suspension, placed on a Bellco roller apparatus, and then incubated at 35 C until confluent cell sheets were obtained (18-24 hr). Once cell confluency was achieved the growth medium was decanted and replaced with serum-free medium 199 containing neomycin and streptomycin as in the growth medium. The CEC roller cultures were held an additional 20-24 hours at 35 C. Prior to infection the medium was removed and the residual fluid drained from the cultures.

Potency Assays. Semliki Forest vaccines were assayed using 3-week-old CD-1 white mice. Groups of 10 mice were inoculated intraperitoneally (IP) on days 0 and 7 with 0.2 ml of 5-fold dilutions of vaccine. Fourteen days after the last vaccine dose the mice were challenged IP with 10³-10⁴ mouse IPLD₅₀ of virulent SF virus. Animals were observed for deaths for 14 days. Titration endpoints were determined by the method of Reed and Muench^{3/}. The potency of a vaccine was expressed as the median effective dose (ED₅₀), i.e. the volume of undiluted vaccine given in each dose of the 2-dose series which protected 50% of the mice from death following challenge.

Eastern equine encephalitis vaccines were assayed in Lakeview strain Golden Syrian hamsters (85-95 gm) (Lakeview Hamster Colony, Newfield, N. J.). Groups of 10 hamsters were inoculated IP on days 0 and 7 with 0.5 ml of 5-fold dilutions of vaccine. Challenges with virulent EEE virus were performed 21 days after the last vaccine dose using 10³-10⁴ hamster IPLD₅₀. Titration endpoints and ED₅₀ values were determined as described for SF vaccines.

Semliki Forest Vaccine. In vaccine production it is advantageous to: (1) use as high a dilution of seed virus as possible to reduce carryover of extraneous material from the seed; (2) employ that dilution of seed virus which results in maximum titers of virus at a convenient harvest time; and (3) use as large a volume of maintenance medium as possible, while still producing a vaccine of high potency.

In a preliminary study it was determined that a multiplicity of inoculum (MOI) of ~ 0.005 (i.e., $0.005 \text{ LD}_{50}/\text{cell}$) would result in a high yield of virus in 18-20 hr using CEC roller cultures maintained with 100 ml of medium 199. Subsequent studies were designed to determine the interrelationships of volume of maintenance medium, virus yield, and time of harvest. To this end replicate CEC roller bottle cultures were each inoculated with 10 ml of a suspension of seed virus to effect an MOI of ~ 0.005 . The bottles were then placed on the roller apparatus for 1 hr at 35°C for virus adsorption. The residual inoculum was then drained from the cultures and either 50, 100, 200 or 300 ml of maintenance medium 199 containing neomycin, streptomycin and 0.25% human serum albumin, was added. All infected cultures were incubated at 35°C on a roller bottle apparatus for 24 hr. (In all subsequent studies described in this report the above procedures were routinely employed for inoculation and handling of cultures.)

TABLE I. EFFECT OF MAINTENANCE MEDIUM VOLUME ON PROPAGATION OF SF VIRUS IN CEC ROLLER BOTTLE CULTURES (MOI = 0.005)

HOUR POST- INFECTION	LOG ₁₀ LD ₅₀ /ml ^a / BY MAINTENANCE MEDIUM VOLUME			
	50 ml	100 ml	200 ml	300 ml
2	3.3	3.1	2.5	2.6
4	3.0	2.6	2.4	2.3
8	5.9	5.4	4.8	4.6
10	6.8	6.0	5.2	5.4
12	7.9	7.2	6.8	6.9
14	8.6	8.1	7.1	7.0
16	9.0	8.5	7.4	7.8
18	8.9	9.5	8.9	8.6
20	9.7	9.9	9.0	9.7
24	9.5	9.4	9.7	9.4

a. Mean titers of samples obtained from replicate sets of cultures.

Table I is a summary of typical virus titers of fluid samples removed from cultures at various times postinoculation. With smaller volumes of maintenance medium (e.g. 50-100 ml) maximum titers were generally achieved in 16-20 hours, whereas with larger volumes 18-24 hr were required. Concomitant with the maximum virus release, cell destruction was greater in cultures maintained with 50-100 ml of medium than with 200 or 300 ml.

Such cell destruction can be expected to increase total protein nitrogen in the final vaccine. Since no data were available to indicate the relationship between final vaccine potency and pre-inactivation virus titer, vaccines were prepared from the 18- 20- and 24-hr fluids from the above studies. Predicated on these results additional SF viral harvests from cultures maintained with 100, 200 or 300 ml of medium 199 were also processed into vaccines.

For final processing into vaccines all harvests were clarified by centrifugation at 900 g for 30 min at 4 C, followed by filtration through a 0.45 μ membrane filter (Millipore). These steps ensured removal of cellular debris which might adversely affect virus inactivation. Studies on numerous lots of SF vaccine have indicated that little or no loss of infectivity occurs as a result of these clarification measures.

Inactivation of clarified viral harvests was accomplished by addition of formalin (Formaldehyde, 37% assay) to a final concentration of either 0.05 or 0.1%. After addition of formalin the fluids were thoroughly mixed by shaking and placed at 37 C for 24-72 hours. During this period the fluids were agitated frequently. At the end of the desired period of inactivation the material was held at 4 C in sealed vessels for 15 days, during which time the vessels were shaken at least twice daily.

TABLE II. FORMALIN INACTIVATION^{a/} OF SF VIRUS AT 37 C.

HOUR POST- FORMALIN	LOG ₁₀ LD ₅₀ /0.03 ml BY FORMALIN CONCENTRATION:		VIRUS ^{b/} CONTROL
	0.05%	0.1%	
0	7.2-8.4	7.6	7.6
2	\leq 3.0-5.3	2.5-3.2	7.2
4	1.3-3.4	0.6-1.2	
6	\geq 0.5-1.5	0-<1.0 _{c/}	
8	\leq 0.5-<1.5	0-<1.0	
10	0-1.5	0-<1.0	
12	0-<1.0	0-<1.0	6.5
14	0-<1.0	0 _{d/}	
16	0-<1.0	0	
18	0	0	
20	0	0	
22	0	0	
24	0	0	6.0

a. Determined by IC inoculation of adult mice with 0.03 ml.

b. Blanks - not tested.

c. When 2 figures are given, this indicates the range observed at that time period on samples from different lots of vaccine.

d. 0 indicates survival of 50/50 mice inoculated with undiluted vaccine.

Samples of vaccine taken at regular intervals during the inactivation period were titrated in mice; samples of viral harvests subjected only to 37 C for similar periods were included as controls. Shown in Table II are representative results of such titrations which have been performed on the majority of vaccines produced.

With 0.05% formalin, "complete inactivation" generally occurred in 10-16 hr. Using a final concentration of 0.1% "complete inactivation" generally occurred in 6-12 hr. The exact "killing time" varied from lot to lot and therefore, must be determined for each individual lot. Since Public Health Service standards^{4/} require formalin treatment at 37 C for 3 times that period required for "complete inactivation," this would entail 30-48 hr inactivation periods for 0.05% vaccines and 18-36 hr for 0.1% vaccines.

Both extended contact with formalin (particularly in higher concentrations) and exposure to temperatures above 0 C may result in decreased antigenicity in certain vaccines, and thus lower potencies. To evaluate the effect of these factors on SF vaccines 15 lots of experimental vaccines were prepared using either 0.05% or 0.1% formalin and inactivation periods of 24-72 hr. Listed in Table III are the results of potency assays performed on vaccines prepared during this study.

TABLE III. EFFECT OF FORMALIN CONCENTRATION AND LENGTH OF INACTIVATION PERIOD ON POTENCY OF SF VACCINES

FORMALIN CONCENTRATION %	ED ₅₀ (ml) BY HOURS OF INACTIVATION:			
	24 hr	30 hr	36 hr	72 hr
0.05	0.0060 ^{a/} (0.018-0.0011)		0.0011	0.0029
0.1	0.0068	0.0036		
	0.0021	0.0033		

a. Mean of 9 lots; (range), all other values are for individual lots of vaccine.

These results indicate that potent SF vaccines may be produced with either 0.05% formalin using a long (e.g. 72 hr) inactivation period or with 0.1% formalin using shorter periods of inactivation. From a production standpoint the latitude thus permitted is of obvious logistical advantage. Also significant, but not shown in Table III, are the volumes of maintenance medium employed on cultures used to produce these vaccines, and the titers of the viral harvests prior to final processing. Maintenance

medium volumes ranged from 50-300 ml, while pre-inactivation titers varied from $10^{9.0}$ - $10^{9.8}$ LD₅₀/ml. No correlation could be made between pre-inactivation titer and vaccine potency. Apparently even the lowest titers achieved represented sufficient antigenic mass for potent vaccines. It is therefore possible to use 300 ml of maintenance medium for high volume production without a concomitant decrease in potency of the final product.

Representative lots of SF vaccines have been lyophilized and are currently being tested for potency. Standard tests^{4/} for safety, toxicity and sterility are also being performed on selected lots.

Eastern Equine Encephalitis Vaccine. Studies are in progress with EEE virus, utilizing the methods described for SF virus.

Roller bottle CEC cultures were infected with PE-6 strain EEE virus at MOI's of 5.0-0.0005 and maintained with 300 ml of medium 199. Samples of culture fluids were removed at regular intervals postinoculation and assayed in mice. As shown in Table IV optimum yields of virus were obtained at a convenient harvest time of 18-24 hr at an acceptable MOI of 0.005.

TABLE IV. EFFECT OF MOI ON PROPAGATION OF EEE VIRUS IN ROLLER BOTTLE CEC CULTURES

HOURS POST- INOCULATION	LOG ₁₀ LD ₅₀ /ml BY MOI:				
	5	0.5	0.05	0.005	0.0005
6	≥7.0	≥6.7	≥7.0	>6.5	5.0
12	≥8.9	>9.0	≥9.0	>8.3	≥8.7
18	9.2	9.0	8.8	8.7	8.7
24	9.0	9.0	9.1	9.2	8.2
30	8.5	8.0	8.7	9.0	≤8.5

Since previous experience in this laboratory suggested that EEE virus yield may be affected by maintenance medium volume, replicate cultures were infected at an MOI of 0.0005 and then maintained with 100-300 ml of medium 199. The results of titrations performed with fluids removed from these cultures at regular intervals are shown in Table V. Although use of 100 or 200 ml of maintenance medium resulted in somewhat higher yields of virus at a convenient harvest time (18-24 hr), the cultures thus maintained showed far greater cytopathic effects (CPE) than those maintained with 300 ml. Thus, the amount of total protein nitrogen can be expected to be greater in the 100- or 200-ml harvests. Assays of additional 18- and 24-hr

TABLE V. EFFECT OF MAINTENANCE MEDIUM VOLUME ON PROPAGATION OF EEE VIRUS IN CEC ROLLER BOTTLE CULTURES (MOI = 0.005)

HOURS POST- INOCULATION	LOG ₁₀ LD ₅₀ /ml BY MAINTENANCE MEDIUM VOLUME:		
	100 ml	200 ml	300 ml
6	7.2	7.0	6.8
12	9.9	9.1	9.1
18	10.0	9.5	9.1
24	10.3	9.8	9.3
30	9.3	9.3	9.3

harvests from cultures maintained with 100-300 ml of media indicate that significantly higher titers are not consistently obtained with the smaller volumes of medium. In all cases, however, titers $\geq 10^{9.0}$ LD₅₀/ml were obtained regardless of volume of the maintenance medium.

Inactivation of clarified viral harvests was carried out at 37 C as described for SF virus, using final concentrations of 0.05, 0.75 or 0.1% formalin for 24-48 hr. Data available at this time suggest the following time requirements for "complete inactivation" of EEE virus: with 0.05% formalin, 8-16 hr; with 0.075%, 6-12 hr; and 0.1%, 4-8 hr.

To date 21 small lots of EEE vaccine have been prepared and are being assayed for potency in hamsters. These vaccines were made with fluids from infected cultures maintained with 100-300 ml of medium and had pre-inactivation virus titers ranging from $10^{9.0}$ - $10^{10.3}$ LD₅₀/ml. Represented in these lots are vaccines prepared with the 3 formalin concentrations employing various periods of inactivation. Until completion of the potency assays it will not be possible to ascertain the importance and interrelationships of factors such as maintenance medium volume, pre-inactivation titer, amount of formalin employed, and length of inactivation period.

Summary:

Fifteen lots of potent Semliki Forest (SF) vaccine have been prepared in roller bottle cultures of chick embryo cells (CEC). The following parameters for SF vaccine production are presented: (1) using a multiplicity of inoculum (MOI) of 0.005, CEC cultures yield maximum titers of virus 18-24 hr postinoculation; (2) ≤ 300 ml of maintenance medium may be employed without decreasing final potency; and (3) inactivation at 37 C may be carried out with 0.05% formalin for 30-72 hr, or 0.1% formalin for 24-30 hr.

Utilizing similar methods the following parameters have been established for EEE vaccine production in CEC roller bottle cultures: (1) a MOI of ≥ 0.005 may be employed; (2) titers $\geq 10^{9.0}$ LD₅₀/ml are consistently achieved by 18-24 hr in cultures maintained with up to 300 ml of medium; and (3) inactivation with 0.05% formalin is complete in 8-16 hr. Potency tests on EEE vaccines are in progress.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 408: Role of Antibody in the Clinical Manifestations of Venezuelan Equine Encephalitis

Reporting Installation: U. S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

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Reports Control Symbol: RCS-MEDDH-288 (R1)

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
				DA OLO811	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8A. DISSEM INSTR*	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES*		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62706A		1B662706A096		02	
b. CONFIDENTIAL		62124011		1B622401A096		02	
c. CONFIDENTIAL		CDOG 1212b(9)					
11. TITLE (Precede with Security Classification Code)*							
(U) Role of antibody in the clinical manifestations of Venezuelan equine encephalitis							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS*							
003500 Clinical Medicine; 004900 Defense; 003200 BW, CW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
62 02		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		0	
b. NUMBER:				FISCAL		0	
c. TYPE: NA				YEAR		5	
d. AMOUNT:				CURRENT		1	
e. KIND OF AWARD:				70		5	
f. CUM. AMT.							
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: USA Medical Research Institute of Infectious Diseases				NAME: Virology Division			
ADDRESS: Fort Detrick, Md 21701				USA Medical Research Institute of Infectious Diseases			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Crozier, D.				NAME: Cole, Jr., F. E.			
TELEPHONE: 301 663-4111 Ext 5233				TELEPHONE: 301 663-4111 Ext 4203			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered.				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Prophylaxis; (U) Encephalitis, equine (VEE); (U) Virus diseases; (U) Immune serum							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Study the role of antibody as it relates to the clinical manifestations of Venezuelan equine encephalitis virus infection, and the usefulness of immune serum in prophylaxis and treatment of this infection.							
24. (U) Animals are inoculated with either attenuated or virulent VEE virus. The efficacy of antiserum in preventing undesirable reactions to these viruses is evaluated. The resulting immune response and its dependency on the relationship of the quantity of antiserum given to time of its administration are investigated.							
25. (U) 68 07 - 69 06 - No work was accomplished during this report period due to lack of personnel. It is anticipated the study will be resumed within the next reporting period.							

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 408: Role of Antibody in the Clinical Manifestations of Venezuelan Equine Encephalitis

Description:

Study the role of antibody as it relates to the clinical manifestations of Venezuelan equine encephalitis virus infection, and the usefulness of immune serum in prophylaxis and treatment of this infection.

Progress and Summary:

No work was accomplished during this report period due to lack of personnel. It is anticipated the study will be resumed within the next reporting period.

Publications:

None.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 409: Host-parasite Relationships in Virus Immunization

Reporting Installation: U. S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

Divisions: Animal Assessment and Pathology

Period Covered by Report: 1 July 1968 through 30 June 1969

Professional Authors: Richard O. Spertzel, Major, VC (II, III)
Charles H. Hobbs, Captain, VC (I, II)
J. Brent Rollins, Captain, VC (I, II)
Frank E. Chapple, III, Captain, VC (III)
Gilberto S. Trevino, Lt Colonel, VC (I, II)

Reports Control Symbol: RCS-MEDDH-288(R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
				DA OL0861	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DES'N INSTR'N	8b. SPECIFIC DATA- CONTRACTOR ACCESS	9. LEVEL OF SUM
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	62706A	1B662706A096		02	409		
b. CH/CH/CH/CH/CH	62124011	1B622401A096		02			
c. CH/CH/CH/CH/CH	CDOG 1212b(9)						
11. TITLE (Precede with Security Classification Code)*							
(U) Host-parasite relationships in virus immunization							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS*							
003500 Clinical medicine; 004900 Defense; 003200 CW, BW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
61 07		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		1	
b. NUMBER:				FISCAL YEAR		60	
c. TYPE:				CURRENT		1	
d. AMOUNT:				70		60	
e. KIND OF AWARD:				f. CUM. AMT.			
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME* USA Medical Research Institute of Infectious Diseases				NAME* Animal Assessment Division			
ADDRESS* Fort Detrick, Md 21701				ADDRESS* USA Medical Research Institute of Infectious Diseases			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Crozier, D.				NAME* Spertzel, R. O.			
TELEPHONE: 301 663-4111 Ext 5233				TELEPHONE: 301 663-4111 Ext 4113			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered.				ASSOCIATE INVESTIGATORS			
				NAME: Hobbs, C. H.			
				NAME: Trevino, G. S. DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Virus disease; (U) Vaccines; (U) Immunization; (U) Yellow fever; (U) Encephalitis (VEE, EEE); (U) Infectious canine hepatitis							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Investigate various interactions between animal hosts and virus vaccines.							
24. (U) Various virus vaccines are given to laboratory animals or man; responses are measured.							
25. (U) 68 07 - 69 06 - The pathogenicity and immunogenicity of small plaque mutant (SPM) strain of Eastern equine encephalitis (EEE) was evaluated in equines. Intracerebral (IC) inoculation of SPM-EEE in a burro produced very mild lesions typical of viral multiplication. Subcutaneous inoculation of SPM produced no significant antibody response and the animals succumbed to an IC challenge with virulent EEE. Dogs infected with virulent infectious canine hepatitis (ICH) virus have shown typical clinical, laboratory and histopathological changes, as well as an increase in alpha-2 fraction of serum protein and glycoproteins. Control animals and animals receiving modified live ICH vaccine have shown no such changes. The interaction of 2 live viral vaccines, Venezuelan equine encephalitis (VEE) and yellow fever, was studied in monkeys. Monkeys that received a combined vaccine inoculum had less response to the yellow fever component when compared to monkeys that received 1 vaccine initially, followed by 3 days by the other. No difference in hemagglutination inhibiting VEE titers was observed.							

* Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)
Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties
Work Unit No. 096 02 409: Host-parasite Relationships in Virus Immunization

Description:

Investigate various interactions between animal hosts and virus vaccines.

Progress, Part I:

Studies were initiated to characterize the pathogenicity and immunogenicity of small plaque mutant (SPM) strain of Eastern equine encephalitis (EEE) virus in equines. Initially, a burro was inoculated with 10^6 median suckling mouse intracerebral lethal doses (SMICLD₅₀) of SPM intracerebrally (IC). The burro was euthanized by injection of Lethal on day 8 postinoculation. During the 8-day observation period, the animal's complete blood count, differential count, packed cell volume, blood urea nitrogen, alkaline phosphatase, serum glutamic oxalacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) values remained within normal limits. No clinical signs typical of central nervous system (CNS) involvement were noted and the animal's temperature remained < 101 F. No viremia was detected.

Gross pathologic examination of the brain revealed a darkly hemorrhagic lesion approximately 1 cm in diameter in the left cerebral hemisphere, directly beneath the trephine aperture in the calvarium. Microscopically, this area was frankly hemorrhagic and focal in nature, apparently due to trauma from injection of the material. Microscopic examination of tissue at increasing distance from this area revealed perivascular cuffing, neuronophagia, tigrolysis of neurons, and satellitosis that could not be ascribed to the surgical trauma alone, and was apparently the result of viral multiplication. However, the mildness of the lesions observed was harmonious with the lack of clinical signs exhibited by the burro.

A burro and a pony were inoculated with 10^6 SMICLD₅₀ SPM subcutaneously (SC). No clinical signs were noted and hematological and blood chemistry parameters remained within normal limits for 36 days. No viremia was detectable in morning and evening samples for the first 8 days postinoculation. Serologic response, as measured by hemagglutination inhibition (HI) titers at various times as long as 36 days postinoculation, was so marginal ($\leq 1:20$) that it was insignificant. At this time, the animals were challenged IC with

10^3 median guinea pig IC lethal doses of PE-6 strain EEE virus. Both succumbed with typical CNS signs of EEE. The duration of illness was only slightly longer than in a control pony.

Summary, Part I:

Studies were initiated to characterize the pathogenicity and immunogenicity of SPM-EEE in the equine species. IC inoculation of SPM in a burro produced mild lesions in the brain typical of viral infection, although there were no clinical signs of CNS involvement. A burro and a pony given SPM SC had no detectable viremia or significant serologic response, and readily succumbed to an IC challenge with virulent EEE.

Progress, Part II:

Studies were initiated to characterize changes in the canine from the effect of modified live infectious canine hepatitis (MLV-ICH) virus and virulent ICH. Clinical observations, daily temperatures, complete blood counts, SGPT, SGOT, alkaline phosphatase, serum protein electrophoresis, glycoprotein electrophoresis, and histopathology (liver biopsy) have been used to investigate this hepatotropic virus.

A total of 21 beagle pups, 4-6 mon old, have been utilized in the following manner:

- a. Liver biopsies.
 - (1) No virus - 2.
 - (2) MLV-ICH - 2.
 - (3) ICH - 1.
- b. Serial bleeding.
 - (1) No virus - 1.
 - (2) MLV-ICH - 7.
 - (3) ICH - 8.

The liver biopsies were performed between days 0 and 6 using the technique of McConnell et al.^{1/} All except 4 dogs were distemper-vaccinated prior to the experiment. All pups for serial bleeding had several baseline bleedings and then were sampled on days 0, 1, 2, 3, 5, 7, 10 and 28.

All dogs that received the MLV-ICH vaccine (commercial product) remained

normal clinically; all parameters mentioned above remained normal. In addition, 3 of these dogs were subsequently challenged with virulent ICH virus (Cornell strain) and remained completely normal. There was no difference between control dogs and the vaccinated group.

Dogs receiving virulent challenge responded with hyperpyrexia, starting between days 2 and 3; leukopenia, beginning about day 5; and increased SGOT and SGPT following leukopenia. Three of 8 dogs died from 6-10 days post-challenge. Histopathology of the serial liver biopsies and of tissues from animals dying from ICH showed typical intranuclear inclusions. There were no lesions attributable to ICH infection in either the control or the vaccinated dogs used for serial liver biopsies.

All dogs that had been challenged with virulent ICH virus, and also had electrophoretic patterns run on their serum, showed an increase in the alpha-2 fraction for both serum and glycoprotein electrophoresis. This change begins between days 3 and 5 and persists to at least day 28. The electrophoretic patterns of control and vaccinated dogs have not shown this dramatic change in the alpha-2 fraction.

Further work is planned to repeat this study and to determine exactly when the rise begins and how long it persists.

Summary, Part II:

Beagle dogs with experimentally induced ICH have shown typical clinical, laboratory and histopathological changes, as well as an increase in the alpha-2 fraction of serum proteins and glycoproteins. Control animals and animals receiving modified live virus vaccine have shown no changes.

Progress, Part III:

The serologic responses of monkeys were examined to determine if 2 live viral vaccines, Venezuelan equine encephalitis (VEE) and yellow fever, interacted antagonistically or synergistically when administered at various times relative to each other. Initially, the following 3 groups of 12 monkeys each were examined:

a. Group I. Lyophilized vaccines (TC-83 strain of VEE and the 17D strain of yellow fever) were reconstituted with the same diluent and administered as a single inoculum.

b. Group II. TC-83 was injected, followed in 3 days by 17D.

c. Group III. 17D was administered, followed in 3 days by TC-83.

Each monkey was bled on day 0 and on days 14 and 28 postvaccination.

TABLE I. SEROLOGICAL RESPONSES TO YELLOW FEVER VACCINE STRAIN 17D.

	LOG NEUTRALIZATION INDICES POSTVACCINATION					
	DAY 14			DAY 28		
	CONDITION ^a			CONDITION		
	I	II	III	I	II	III
	<1	1.6	1.9	2.5	3.2	2.1
	<1	1.3	<1	1.8	2.0	1.0
	1.7	1.0	1.7	2.9	2.0	2.3
	<1	1.1	1.9	1.7	2.0	2.0
	<1	<1	1.8	1.8	1.5	2.9
	<1	1.6	2.0	<1	1.7	2.0
	<1	1.8	2.1	<1	1.9	2.3
	<1	1.3	1.9	<1	2.0	1.9
	<1	1.9	1.9	<1	1.9	2.3
	<1	<1	1.9	<1	1.5	2.2
	-	<1	1.3	2.3	2.0	2.1
	<1	1.4	1.5	-	1.0	2.5
Summary						
No. Responding/ Total	1/11	9/12	11/12	6/11	12/12	12/12
Mean LNI Titer	0.15	1.08	1.66	1.18	1.88	2.12
Mean LNI Titer Responders	1.68	1.44	1.81	2.17	1.88	2.12

a. I. VEE and 17D vaccines, combined.

II. VEE vaccine, followed in 3 days by 17D vaccine.

III. 17D vaccine, followed in 3 days by VEE vaccine.

TABLE II. SEROLOGIC RESPONSES TO VEE VACCINE.

	RECIPROCAL HI TITER POSTVACCINATION					
	DAY 14			DAY 28		
	CONDITION ^a /			CONDITION		
	I	II	III	I	II	III
	80	80	320	320	40	320
	80	40	160	80	80	160
	320	640	160	320	1280	80
	160	80	320	160	40	320
	160	160	160	160	320	160
	80	640	640	40	160	320
	160	640	320	160	640	160
	20	160	1280	40	160	1280
	160	320	640	80	320	640
	80	160	640	80	320	320
	160	320	320	320	320	320
	320	320	40	320	320	80
<u>Summary</u>						
No. Responding/ Total	12/12	12/12	12/12	12/12	12/12	12/12
Mean HI Titer	120	214	302	135	214	254

a. See footnote Table I.

Yellow fever antibody neutralization tests and VEE-HI tests were conducted on each sample by Serology Section, Bacteriology Division, USAMRIID. The yellow fever antibody neutralization test performed was the constant serum-varying virus technique. Results obtained from these tests are shown in Tables I and II.

From Table I, it can be seen that a significantly smaller percentage of the Group I monkeys developed a demonstrable log neutralization index (LNI) antibody response to 17D than did the monkeys of Groups II and III. This observation is true for both days 14 and 28. Although in Table II a suggested difference in VEE-HI titers exists between Group I and Groups II and III, there is no difference in number of animals responding, and the difference in titer is not significant.

In all cases (both VEE and yellow fever), the prevaccination titers were negative.

Summary, Part III:

The interaction of 2 live viral vaccines, VEE and yellow fever, was studied by serological methods in the monkey. The serological response to yellow fever vaccine was less in a group of monkeys that received the 2 vaccines combined in a single inoculum than in monkeys that received 1 of the vaccines initially, followed in 3 days by the other. No difference was observed in HI titers for VEE among the 3 groups. A more definitive study is now being conducted.

Publications:

None.

LITERATURE CITED

1. McConnell, S., H. W. Whitford, R. D. Feigen, R. D. Harting and R. A. Vandercook. 1969. Use of a plastic window to obtain serial liver, spleen and kidney biopsies in the rhesus monkey. *Southwestern Veterinarian* 22:2, pp ?.

ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 410: Pathophysiology and Treatment of Yellow Fever

Reporting Installation: U. S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

Divisions: Animal Assessment and Pathology

Period Covered by Report: 1 July 1968 through 30 June 1969

Professional Authors: Frank E. Chapple, III, Captain, VC
Richard O. Spertzel, Major, VC
Jerry D. Weil, Captain, VC
Gilberto S. Trevino, Lt Colonel, VC

Reports Control Symbol: RCS-MEDDH-288(R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OL0877	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8a. DISSEM INSTR ^a	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES: ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER		WORK UNIT NUMBER		
a. PRIMARY	62 706A	1B662 706A096	02		410		
b. CONTINUING	62 124011	1B622 401A096	02				
c. CONTINUING	CDOG 1212b(9)						
11. TITLE (Precede with Security Classification Code) ^a							
(U) Pathophysiology and Treatment of yellow fever							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
003500 Clinical Medicine; 004900 Defense; 003200 CW, BW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
66 12		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: ^a				FISCAL		3	
c. TYPE: NA				YEAR		30	
d. AMOUNT:				CURRENT		3	
e. KIND OF AWARD:				70		30	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a USA Medical Research Institute of Infectious Diseases				NAME: ^a Animal Assessment Division			
ADDRESS: ^a Fort Detrick, Md 21701				USA Medical Research Institute of Infectious Diseases			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Crozier, D.				NAME: ^a Chapple, F. E.			
TELEPHONE: 301 663-4111 Ext 5233				TELEPHONE: 301 663-4111 Ext 5215			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered.				ASSOCIATE INVESTIGATORS			
				NAME: Spertzel, R. O.			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Yellow fever; (U) Therapy; (U) Cross-circulation							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Study cross-circulation as a mode of therapy for yellow fever.							
24. (U) Inoculate rhesus monkeys with Asibi strain yellow fever virus. Study changes in blood and tissues during the course of the disease. Study cross-circulation as a mode of therapy.							
25. (U) 68 07 - 69 06 - Cross-circulation of yellow fever-infected monkeys with immune donor animals produces a temporary improvement clinically, but has failed to prolong the life of the animal. Many technical problems have been encountered and are under investigation.							

^a Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 02 410: Pathophysiology and Treatment of Yellow Fever

Description:

Study cross-circulation as a mode of therapy for yellow fever in the rhesus monkey.

Progress:

The study of cross-circulation as a means of therapy in yellow fever in the rhesus monkey has been continued in a manner similar to that outlined previously.^{1/} The various controls discussed in that report have not been used in order to reduce the number of monkeys required for each study. These controls will be reintroduced into the study if an infected animal can be maintained and its life extended for a significant period of time.

During each cross-circulation study, 3-20 ml of blood are drawn at hourly intervals from each monkey and replaced with equal volumes of blood from normal monkeys. The following laboratory studies are performed on each blood sample: viremia, lactic dehydrogenase (LDH), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase, pH, pCO₂, base excess, Na, K and blood urea nitrogen (BUN). In all of the studies where yellow fever-infected monkeys were cross-circulated with immune donor monkeys, the Na, K, alkaline phosphatase, pH, pCO₂, and base excess values have remained relatively unchanged except for the terminal samples. Slight rises in BUN levels have been noted, especially in infected animals and donor animals that have died. The LDH and SGPT values have increased in both the infected and donor animals as the cross-circulation has progressed. However, whereas the infected animal levels remained elevated, or continued to rise, the levels in the donor animals when removed from cross-circulation tended to return to normal. Terminally, metabolic acidosis and hypoglycemia occur in the infected animal.

Five studies have been conducted utilizing yellow fever-infected animals and immune donor animals. As these studies have progressed, the schedules of the cross-circulation periods have been altered in an attempt to find a schedule beneficial to the infected animal. The original schedule was 1 hr cross-circulation followed by a 5-hr rest. The rest period was later reduced to 3 hr. Since this schedule also failed to prolong life, it was decided to

keep the infected animal on continuous cross-circulation, but to change the donor animal every 5 hr. As clotting problems often occurred during the changing of donors, in the last study the infected animal was continuously cross-circulated with 1 donor.

To date, none of the cross-circulation regimes used have prolonged the life of any infected animals. However, many technical problems occur during cross-circulation which may contribute to the deaths of the infected animals. Studies are now in progress to elucidate the mechanisms of these problems and methods of overcoming them.

The most difficult problem to overcome is the clotting in the extracorporeal shunts. This appears to be a greater problem in the infected animal than in the donor animal, or in the case of cross-circulation of 2 monkeys. To substantiate this supposition, we are now in the process of cross-circulating groups of normal monkeys, 2 animals per group. These are cross-circulated continuously for 30 hr and observed for the development of clots in the system. Also, various laboratory procedures are performed in order to detect any adverse changes which may be occurring.

Another problem which is being closely studied is the death of the immune donor animals. Of the 11 donors used in the 5 study groups, 8 have died 24-72 hr following cross-circulation. Of these 8 donors, only 1 has had a demonstrable viremia at the time of death. By working closely with Pathology Division, USAMRIID, we hope to determine the cause of any donor animal deaths in future studies.

In most studies the infected animal initially has shown clinical improvement when placed on cross-circulation. To date, this improvement has only been transient.

Summary:

The use of cross-circulation as a means of therapy for yellow fever is being investigated in the rhesus monkey. To date, the technique has failed to prolong the life of infected animals. However, the many technical problems encountered when cross-circulation is occurring may be contributing to the deaths of the animals and are now under investigation.

Publications:

None.

LITERATURE CITED

1. U. S. Army Medical Unit. 1 July 1968. Annual Progress Report, FY 1968. p. 181 to 184. Fort Detrick, Maryland

ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 03: Laboratory Identification of Biological Agents

Work Unit No. 096 03 002: Identification of Microbial Pathogens by
Fluorescent Antibody Techniques

Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland

Division: Pathology

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Author: Joseph F. Metzger, Colonel, MC

Reports Control Symbol: RCS-MEDDH-288(R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
				DA OL0849	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES:*		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
						WORK UNIT NUMBER	
a. PRIMARY		62706A		1B662706A096		03	
b. CONFIDENTIAL		62124011		1B622401A096		03	
c. CONFIDENTIAL		CDOG 1212b(9); 1412a(2)					
11. TITLE (Precede with Security Classification Code)*							
(U) Identification of microbial pathogens by fluorescent antibody techniques							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS*							
003500 Clinical Medicine; 004900 Defense; 003200 CW, BW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
61 08		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (In thousands)	
b. NUMBER:*				FISCAL YEAR			
c. TYPE:				CURRENT			
d. KIND OF AWARD:							
e. CUM. AMT.							
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME:*				NAME:*			
USA Medical Research Institute of Infectious Diseases				USA Medical Research Institute of Infectious Diseases			
ADDRESS:*				ADDRESS:*			
Fort Detrick, Md 21701				Fort Detrick, Md 21701			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME:				NAME:*			
Crozier, D.				Metzger, J. F.			
TELEPHONE:				TELEPHONE:			
301 663-4111 Ext 5233				301 663-4111 Ext 6206			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered.							
22. KEYWORDS (Precede EACH with Security Classification Code)				ASSOCIATE INVESTIGATORS			
(U) Fluorescent antibody techniques; (U) Pathogens; (U) Rapid identification;				NAME:			
(U) Diagnosis				NAME:			
23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) Study the adaptation of fluorescent antibody techniques to the demonstration of microbial pathogens in animal tissues.							
24 (U) Demonstrate infectious agents or their products utilizing immunofluorescence.							
25 (U) 68 07 - 69 06 - During the current fiscal year previously prepared antisera will be tested and new sera will be prepared.							

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 03: Laboratory Identification of Biological Agents

Work Unit No. 096 03 002: Identification of Microbial Pathogens by
Fluorescent Antibody Techniques

Description:

Study the adaptation of fluorescent antibody techniques to the demonstration of microbial pathogens in animal tissues.

Progress:

During the current year antisera previously prepared were tested and new sera are being prepared.

Publications:

None.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)
Task No. 1B662706A096 03: Laboratory Identification of Biological Agents
Work Unit No. 096 03 003: Diagnosis of Viral Infections with Homologous
Bone Marrow Cultures
Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland
Division: Virology
Period Covered by Report: 1 July 1968 to 30 June 1969
Professional Author: Francis E. Cole, Jr., Ph.D.
Reports Control Symbol: RCS-MEDDH-288 (R1)
Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OL0850	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DISB'N INSTR ^a	9b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES: ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62706A		1B662706A096		03	
b. CONFIDENTIAL		62124011		1B622401A096		03	
c. CONFIDENTIAL		CDOG 1212b(9); 1412a(2)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) Diagnosis of viral infections with homologous bone marrow studies							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
003500 Clinical Medicine; 004900 Defense; 003200 BW, CW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
65 03		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		0	
b. NUMBER: ^a				FISCAL YEAR		b. FUNDS (in thousands)	
c. TYPE:				CURRENT		5	
d. AMOUNT:				70		1	
e. KIND OF AWARD:				f. CUM. AMT.			
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a USA Medical Research Institute of Infectious Diseases				NAME: ^a Virology Division			
ADDRESS: ^a Fort Detrick, Md 21701				ADDRESS: ^a USA Medical Research Institute of Infectious Diseases			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Crozier, D.				NAME: ^a Cole, Jr., F. E.			
TELEPHONE: 301 663-4111 Ext 5233				TELEPHONE: 301 663-4111 Ext 4203			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered.				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
22. KEY WORDS (Precede EACH with Security Classification Code)							
(U) Bone marrow; (U) Encephalitis, equine (VEE); (U) Arbovirus infection; (U) Tissue culture							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Develop a diagnostic system in which bone marrow serves as both a clinical specimen and a host system for in vitro virus propagation; determine the duration of bone marrow infection in animals inoculated with the attenuated strain of VEE virus; define the correlation between persistence of infection and maintenance of antibody level.							
24. (U) Canine hosts are inoculated with attenuated or Trinidad strain VEE virus. At various intervals postinoculation the animals are bled for viremia and hemagglutination-inhibition antibody assays, and samples of marrow are aspirated from the ilium for use in cultural procedures. After incubation for periods up to 10 days at 37 C, virus content of culture fluids is assayed by the mouse protection test.							
25. (U) 68 07 - 69 06 - Due to shift of personnel to work of higher priority, no studies were conducted. Work will resume when sufficient personnel are available.							

^a Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 03: Prevention and Treatment of Biological Warfare Casualties

Work Unit No. 096 03 003: Diagnosis of Viral Infections with Homologous Bone Marrow Cultures

Description:

Develop a diagnostic system in which bone marrow serves as both a clinical specimen and a host system for in vitro virus propagation; determine the duration of bone marrow infection in animals inoculated with attenuated Venezuelan equine encephalitis virus; define the correlation between persistence of infection and maintenance of antibody level.

Progress and Summary:

Due to shift of personnel to work of higher priority, no studies were conducted. Work will resume when sufficient personnel are available.

Publications:

None.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 03: Laboratory Identification of Biological Agents

Work Unit No. 096 03 006: Early Immune Response in Infectious Disease
and Toxemia

Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland

Divisions: Bacteriology and Medical

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Authors: Charles P. Craig, Captain, MC
William S. Irvin, Major, MC

Reports Control Symbol: RCS-MEDDH-288(R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OL0870	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMRY ^a	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8A. DISSEM INSTR ^a	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER		WORK UNIT NUMBER		
a. PRIMARY	62706A	1B662706A096	03		006		
b. Secondary	62124011	1B622401A096	03				
c. Supporting	CDOG 1212b(9); 1412a(2)						
11. TITLE (Precede with Security Classification Code) ^a							
(U) Early immune response in infectious disease and toxemia							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
003500 Clinical Medicine; 004900 Defense; 003200 BW, CW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
66 08		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: ^a				FISCAL YEAR		1	
c. TYPE:				CURRENT		7	
d. AMOUNT:				70		1	
e. KIND OF AWARD:						7	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a USA Medical Research Institute of Infectious Diseases				NAME: ^a Medical Division			
ADDRESS: ^a Fort Detrick, Md 21701				ADDRESS: ^a USA Medical Research Institute of Infectious Diseases			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Crozier, D.				NAME: ^a Irwin, W. S.			
TELEPHONE: 301 663-4111 Ext 5172				TELEPHONE: 301 663-4111 Ext 6130			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered				ASSOCIATE INVESTIGATORS			
				NAME: Craig, C. P.			
				NAME:			
22. KEYWORDS (Precede Each with Security Classification Code)							
(U) Immunology; (U) Antibody formation; (U) Chemotaxis; (U) Hemagglutination;							
(U) Complement; (U) Macrophage; (U) Enterotoxin; (U) Staphylococcus							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Develop and employ sensitive methods for detection of circulating immunoglobulin early in the course of infectious disease.							
24. (U) Sensitivity of various assay techniques for detection of immunoglobulin, including chemotaxis, augmented hemagglutination, and macrophage migration will be determined. These will be applied to detection of circulating antibody and antibody forming cells early in the course of infection in animals and humans.							
25. (U) 68 07 - 69 06 - Studies with purified complement components were not continued due to failure of a -70 C freezer which resulted in loss of activity of the fractions. No work was done on coating particles with antigen. Efforts were expanded on chemotaxis generation of polymorphonuclear leukocytes by staphylococcal enterotoxin B (SEB). A heat-sensitive factor generated by SEB in human serum correlates inversely with a negative precipitin titer and an absence of IgM antibody. Preliminary work was begun on a study of the effect of SEB on macrophage migration.							

^aAvailable to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)
Task No. 1B662706A096 03: Laboratory Identification of Biological Agents
Work Unit No. 096 03 006: Early Immune Response in Infectious Disease
and Toxemia

Description:

Develop and employ sensitive methods for detection of circulating immunoglobulin early in the course of infectious disease.

Progress and Summary, Part I:

Studies reported last year concerning purified complement components were not continued. Early in this fiscal year, a -70 C freezer failed; the purified serum fractions stored there lost all their activity. The C'la transfer assay was therefore discontinued. No work was done on the coating of particles with antigen which had been started a year ago.

Efforts were expanded on the generation of chemotaxis of polymorphonuclear leucocytes (PMN) by staphylococcal enterotoxin B (SEB).

Progress, Part II:

Chemotactic Activity for Polymorphonuclear Leucocytes Generated by SEB.
The migration of PMN across a millipore filter was used to demonstrate the generation of a chemotactic factor by SEB. The technique used was that of Snyderman, et al.^{1/} No activity for rabbit peritoneal PMN was generated by SEB in rat sera, but endotoxin did generate a chemotactic factor in this test system. Results using rabbit sera and rabbit peritoneal PMN were highly variable, but indicated that there was a chemotactic factor generated by SEB. The studies were then extended to fresh human autologous sera and peripheral blood PMN. Typical protocols employing 3 donors with no detectable antibody to SEB are presented in Table I. It can be seen that there is a heat-sensitive chemotactic factor generated by SEB in human sera after incubation at 37 and occasionally at 0 C. In other experiments, heating the serum at 56 C reveals that the substrate is heat sensitive.

TABLE I. CHEMOTACTIC ACTIVITY GENERATED BY SEB OR ENDOTOXIC LIPOPOLY-SACCHARIDE (LPS) IN HUMAN SERUM IN TWO EXPERIMENTS.

INCUBATION		CELLS/10 HIGH-POWER FIELDS					
Temp. & Time	Condition	Donor M		Donor C		Donor I	
		#1	#2	#1	#2	#1	#2
0 C 30 min + 37 C 60 min	SEB 4 μ g	423**	1423*	298**	709**	873**	668*
	LPS 4 μ g	480**	1010**	450*	450*	938**	724*
	Control	243	678	159	358	652	109
0 C 30 min + 37 C 60 min + 56 C 30 min	SEB 4 μ g	197	459	302*	188	468	73
	LPS 4 μ g	494*	612**	450*	623*	840	358*
	Control	240	383	110	203	652	100
0 C 120 min	SEB 4 μ g	237	717**	278**	400	805	382*
0 C 180 min	SEB 4 μ g	247	626	463*	931**	899**	390**
0 C 180 min	Control	242	513	170	358	568	180

* Differs significantly from control value, $p < 0.05$

** Differs significantly from control value, $p < 0.01$

Table II summarizes the data on sera from 8 human donors with varying patterns of anti-SEB antibody. Serum was taken from each donor on 2 separate occasions. As can be seen from the table, failure of SEB to activate a chemotactic factor is associated with a positive precipitin test and the lack of demonstrable IgM as determined by Sephadex G-200 chromatography. No correlation is seen between antibody measurements and the generation of a chemotactic factor at 0 C. No explanation for the generation of a chemotactic factor at this temperature is apparent. The heat lability and the association of the presence of precipitating antibody with protection against challenge with SEB indicate that this chemotactic factor is different from that generated by endotoxin and antigen-antibody complexes. Preliminary experiments indicate the chemotactic factor is stable for 12-24 hr. The parallelism between species susceptibility to SEB and the generation of chemotactic activity in their sera suggests a possible role of the chemotactic factor in the pathogenesis of SEB intoxication.

TABLE II. EFFECT OF ANTI-SEB TITERS OF SERA ON CHEMOTACTIC ACTIVITY IN SEB-HUMAN SERUM MIXTURES.

DONOR	RECIPROCOL TITERS		ECA TITER μg/ml neutralized	ACTIVITY AFTER INCUBATION		IgM ANTIBODY BY G-200 SEPARATION
	Hemagglutinin	Precipitin		37 C	0 C	
Ja	5120	0	0	+,+ ^{a/}	-, -	-
Ol	2560	0	0.5	+,+	-, -	-
Jo	10000	4	2.5	-, -	+,+	+
Sh	5120	4	2.5	-, -	+,+	+
Di	5120	8	6.0	-, -	+,+	+
Ha	2560	8	2.5	-, -	-, -	+
Ty	1280	2	2.5	-, -	-, -	+
Ku	10000	8	3.0	-, -	-, -	+

a. + = Significantly greater than controls; - = no significant difference

Summary, Part II:

Three possible mechanisms by which a chemotactic factor might be generated in serum-SEB mixtures are: (1) direct action of SEB on a special serum substrate; (2) combination of SEB with some serum component; (3) fragmentation of SEB by some serum enzyme or other substance to give an active breakdown product. Studies using mixtures of fluorescein labelled SEB and serum, chromatographed on Sephadex G-25, -50 and -75 columns, gave no evidence to support the last 2 hypotheses.

Progress, Part III:

Effect of SEB on Macrophage Migration. It has been demonstrated that the reaction to intradermal injection of SEB and endotoxin mimics delayed type hypersensitivity in rabbits.^{2/} Furthermore, Heilman and Bast have shown that the migration of macrophages from a spleen explant is inhibited by endotoxin.^{3/} SEB and endotoxin have both been shown to alter function of the reticuloendothelial system (RES) in vivo.^{4/} Finally, death from SEB intoxication is usually a delayed response occurring 1-5 days after injection. For these reasons, the technique of David, et al,^{5/} using the migration of peritoneal macrophages from a capillary tube was applied to the study of SEB and endotoxin. This technique offers the obvious

advantage of in vitro manipulation of the variables and allows the comparison of mixed peritoneal populations with purified macrophages.

The results are expressed as per cent of the area covered by cells migrating from test capillaries compared with that of cells from the same animals in capillaries with no toxin added. Most of the data reflect the average of 4 tubes in 2 separate plastic petri dishes. In multiple duplicate controls, using one cell preparation, there is <1 chance in 30 that the average migration of one petri dish (2 capillaries) will fall below 80% of the migration of the average of all of the control tubes in that experiment. Based on this observation, a migration that averages 75% or less of the control is considered to be significantly inhibited.

Table III summarizes the data from adult animals. The mean values show a typical dose response curve with inhibition occurring at a concentration of 0.1 μg SEB/ml. The number of animals responding with significant inhibition at each concentration is also tabulated. At 100 $\mu\text{g}/\text{ml}$, 16 of 22 animals tested responded with migration \leq 75% of that of the controls. A typical dose response curve is shown in the last column of the table. The macrophages from some animals fail to respond to even 100 $\mu\text{g}/\text{ml}$, and a rare animal will respond to as little as 0.01 $\mu\text{g}/\text{ml}$. All animals were negative for antibody to SEB. Thus, prior immunization does not account for the variability.

TABLE III. EFFECT OF SEB ON THE MIGRATION OF GUINEA PIG MACROPHAGES

AMOUNT SEB $\mu\text{g}/\text{ml}$	\leq 75% OF CONTROL No./Total	% OF AREA OF CONTROL MIGRATION		
		Mean	Range	A Typical Dose Response
100	16/22	55	20-118	20
10	13/18	64	28-116	28
1	11/17	66	40-97	40
0.1	9/16	73	45-95	45
0.01	1/10	86	72-98	90
0.001	1/7	99	75-130	

Table IV gives similar data for endotoxin. Fewer animals have been tested, but it is apparent that using a single lot of Escherichia coli endotoxin inhibition is observed consistently only at a concentration of 100 $\mu\text{g}/\text{ml}$.

TABLE IV. EFFECT OF ENDOTOXIN ON THE MIGRATION OF PURIFIED GUINEA PIG MACROPHAGES

AMOUNT ENDOTOXIN $\mu\text{g/ml}$	< 75% OF CONTROL No./Total	% OF AREA OF CONTROL MIGRATION	
		Mean	Range
100	11/14	53	17-92
10	2/10	83	33-104
1	3/11	87	58-127
0.1	2/9	87	56-119
0.01	0/6	97	88-112
0.001	0/3	100	88-120

The next set of experiments was designed to determine the site of action leading to the inhibition of macrophage migration. Animals sensitive to bovine gamma globulin (BGG) were used. Both mixed populations and purified macrophages were tested. The separation technique employed yielded 99.9% pure macrophages (by morphologic criteria). The cells were not inhibited by addition of BGG to the medium and hence, were functionally pure, since the only known immunologic mechanism, mediated by sensitized lymphocytes, for inhibition of macrophage migration was inoperative. Bovine pancreatic RNase was included as an unrelated basic protein control. Only 6 technically acceptable experiments have been completed; the results of these are presented in Table V (others with contamination, low cell counts, etc., were eliminated). Although these studies are preliminary, the data suggest a dual action of high concentrations of SEB in this system. Though inhibition is not as marked with purified cells as in mixed populations, it is markedly greater than control values, suggesting that SEB acts both directly on macrophages and also on some cell in the peritoneal exudate, which in turn augments the inhibition of macrophage migration. In 5 experiments using a purified macrophage population and concentrations of less than 100 μg SEB/ml, no inhibition has been demonstrated. Although the available data do not rule out a modest inhibition by a negatively charged protein such as RNase, it seems clear, by comparison of results, that the negative charge per se does not account for the action of SEB.

In other preliminary experiments, using the macrophage migration system, it has been found that: (1) cells from an adult female guinea pig are more sensitive to SEB than are the macrophages from her new-born young, and (2) cells from immunized animals are more sensitive to SEB than those from unimmunized animals.

TABLE V. EFFECT OF SEB AND ENDOTOXIN ON MIXED AND PURE MACROPHAGE MIGRATION

ANIMAL NO.	% OF AREA OF CONTROL MIGRATION							
	SEB		ENDOTOXIN		BGG-Sensitized		RNase Control	
	100 μ g/ml	100 μ g/ml	100 μ g/ml	100 μ g/ml	20 μ g/ml	20 μ g/ml	200 μ g/ml	200 μ g/ml
	Mixed	Pure	Mixed	Pure	Mixed	Pure	Mixed	Pure
1	21	37	-	-	45	110	-	-
2	36	61	36	-	48	89	96	-
3	39	77	35	66	58	93	88	99
4	54	42	40	77	44	92	92	88
5	31	105	38	102	45	108	74	98
6	22	52	-	-	41	102	60	-
Mean	34	62	37	82	47	99	82	95

Experiments of similar design are being initiated with rabbit macrophages.

Summary, Part III:

SEB has been found to inhibit the migration of macrophages from capillary tubes. Results of work reported suggest a dual action of SEB. At high concentrations, it acts on the macrophage itself, while at lower concentrations inhibition of macrophage migration of SEB must be augmented by its action on some other cell in the peritoneal exudate. The action of SEB on the macrophage in vitro is not due to its negative charge per se. At high concentrations, the action of SEB is not mediated through the known immunologic mechanism of inhibiting macrophage migration.

Cells from new-born animals are less sensitive to SEB than cells from older animals. Specific immunization increases the sensitivity of cells to SEB.

Publications:

None

LITERATURE CITED

1. Snyderman, R., H. Gewurz, and S. E. Mergenhagen. 1968. Interactions of the complement system with endotoxic lipopolysaccharide. Generation of a factor chemotactic for polymorphonuclear leucocytes. J. Exp. Med. 128:259-275.
2. Carozza, Jr., F. A. March 1967. Mechanisms of pyrogenicity of staphylococcal enterotoxin B, p. 43 to 55. In Commission on Epidemiological Survey. Annual Report to The Armed Forces Epidemiological Board, Fiscal Year 1966. (AD 810 278L).
3. Heilman, D. H., and R. C. Bast, Jr. 1967. In vitro assay of endotoxin by the inhibition of macrophage migration. J. Bact. 93:15-20.
4. Sugiyama, H. 1966. Endotoxin-like responses induced by staphylococcal enterotoxin. J. Inf. Dis. 116:162-170.
5. David, J. R., S. Al-Askari, H. S. Lawrence, and L. Thomas. 1964. Delayed hypersensitivity in vitro. I. The specificity of inhibition of cell migration by antigens. J. Immun. 93:264-273.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 03: Laboratory Identification of Biological Agents

Work Unit No. 096 03 007: Peritoneal Eosinophilia as an Assay for
Antigen-antibody Complexes

Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland

Division: Bacteriology and Medical

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Author: William S. Irvin, Major, MC

Reports Control Symbol: RCS-MEDDH-288(R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL	
				DA OL0887	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISPN INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES*		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62706A		1B662706A096		03	
b. Continuation		62124011		1B62401A096		03	
c. Continuation		5DOG 1212b(9);		1412a(2)			
11. TITLE (Precede with Security Classification Code)*							
(U) Peritoneal eosinophilia as an assay for antigen-antibody complexes							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS*							
003500 Clinical, Medicine; 004900 Defense; 003200 CW, BW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
68 01		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER:				FISCAL		69	
c. TYPE:				YEAR		CURRENT	
d. KIND OF AWARD:				70		1	
e. CUM. AMT.						5	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: USA Medical Research Institute of Infectious Diseases				NAME: Bacteriology Division			
ADDRESS: Fort Detrick, Md 21701				USA Medical Research Institute of Infectious Diseases			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Crozier, D.				NAME: Irvin, W. S.			
TELEPHONE: 301 663-4111 Ext 5233				TELEPHONE: 301 663-4111 Ext 6130			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered.				ASSOCIATE INVESTIGATORS			
				NAME: DA			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Eosinophilia; (U) Antigens; (U) Antibodies; (U) Guinea pigs; (U) Antigen-antibody complexes							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Determine the feasibility of using induced peritoneal eosinophilia as an assay for antigen-antibody complexes present in body fluid.							
24. (U) Induce eosinophilia by injecting intraperitoneally (IP) a noncross-reacting protein antigen into guinea pigs. Follow with IP injection of antigen-antibody complexes and measure the effect on the number of eosinophils.							
25. (U) 68 07 - 69 06 - Using an antiserum containing a large amount of antibody, the eosinophilotactic properties of antigen-antibody complexes have been confirmed. The usefulness of the technique for these complexes is questioned. Attempts thus far to develop a more sensitive <u>in vitro</u> technique have been unsuccessful.							

*Available to contractors upon originator's approval.

JOD, DPG

27

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)
Task No. 1B662706A096 03: Laboratory Identification of Biological Agents
Work Unit No. 096 03 007: Peritoneal Eosinophilia as an Assay for
Antigen-antibody Complexes

Description:

Determine the feasibility of using induced peritoneal eosinophilia as an assay of antigen-antibody complexes in body fluid.

Progress:

After establishing the baseline peritoneal eosinophilia in Fort Detrick guinea pigs, attempts were made to repeat the work of Litt.^{1/} He stated that eosinophil counts in most animals fell to baseline 1 week after the last injection of hemocyanin, but we were unable to confirm this observation. Washing the animal's peritoneal cavity once or twice weekly for 2 weeks following the last hemocyanin injection failed to produce a satisfactory baseline.

The next experiment added a 3rd week of wash and included an injection of balanced salt solution (BSS) on the 3rd week. In this way, it was hoped to reduce the background further and to be able to use each animal as his own control by comparing the response to antigen-antibody complexes as a per cent of the response to BSS. This experiment involved 20 animals; 6 BSS controls, 5 antigen controls, 5 antibody controls, and 4 animals receiving antigen-antibody complexes as the test. Analysis of the data by determining the total number of cells removed, the total number and per cent of eosinophils removed, and comparison with values the previous weeks' response showed complete overlap of the control and antigen-antibody complex groups.

In the next experiment, the animals were washed daily starting 24 hr after the 6th weekly injection of hemocyanin until the total number of eosinophils removed was less than the mean background count + 2 SD (8.7×10^6 eosinophils).

Four hours later, animals were challenged with the test substance; in 24 hr the peritoneal cavity was washed; the total number of eosinophils was counted. The combined results of 4 separate experiments are presented in Table I. The antiserum had 240 μ g of antibody N/ml. The complexes were made in antigen excess (2-3x). Each test substance was injected in a volume of 1 ml. An increase of $\geq 2.5 \times 10^6$ eosinophils/over the background count was considered a positive response; their numbers are shown at the bottom of Table I. Sixty per cent of the animals tested with antigen-antibody complexes were positive. None of the antigen or antibody controls,

TABLE I. EOSINOPHIL RESPONSE TO ANTIGEN-ANTIBODY COMPLEXES AND CONTROLS

MILLIONS OF EOSINOPHILS IN THREE 15 ml WASHES BY SUBSTANCE TESTED											
Balanced Salt Solution			Antigen		Antibody		Antigen-Antibody Complexes				
Eosinophils 24 hrs Before or After	Δ	Eosinophils 24 hrs Before or After	Δ	Eosinophils 24 hrs Before or After	Δ	Eosinophils 24 hrs Before or After	Δ				
6.2	1.4	-4.8	4.4	3.2	-1.2	7.0	4.1	-2.9	0.9	1.7	+0.8
4.8	2.5	-2.3	1.7	2.5	+0.8	1.4	0.8	-0.6	0.1	3.1	+3.0
0.0	0.3	+0.3	0.6	0.2	-0.4	0.7	1.2	-0.5	0.5	1.8	+1.3
3.5	1.1	-2.4	0.8	0.0	-0.8	0.9	0.1	-0.8	0.0	5.2	+5.2
6.5	2.3	-4.2	2.3	1.8	-0.5	4.4	0.8	-3.6	2.4	9.8	+7.4
6.8	1.3	-5.5	3.3	2.0	-1.3	1.5	1.6	+0.1	3.4	4.6	+1.2
3.3	2.1	-1.2	0.2	1.4	+1.2	5.6	0.8	-4.8	0.2	10.8	+10.6
7.7	6.9	-1.2				4.1	5.5	+1.4	6.8	10.7	+3.9
3.7	9.1	+5.4				6.9	2.1	-4.8	0.9	0.5	-0.4
						3.3	4.6	+1.3	1.6	0.3	-1.3
						0.1	0.2	+0.1	1.7	1.2	-0.5
						0.8	0.1	-0.7	4.9	8.2	+3.3
						0.5	0.7	+0.2	3.5	8.6	+5.1
						4.5	2.7	-2.7	4.0	18.3	+14.3
									5.0	22.1	+17.0
									5.5	2.2	-3.3
									6.5	11.2	+4.7
									3.8	8.3	+4.5
									4.1	4.5	+0.4
									2.4	14.8	+12.4
No. Positive/Total											
with increase											
> 2.5 x 10 ⁶											
1/9			0/7		0/14		12/20				

but one of 9 BSS controls gave a positive response. During the course of these experiments an antitetanus toxoid antiserum containing approximately 60 μ g of antibody N/ml was used in an attempt to test the lower limits of the sensitivity of the technique. The antiserum had been stored for 1 yr at 4 C, 2 of 3 controls gave a positive response. This result was interpreted as resulting from change secondary to prolonged storage. New antiserum is being collected. Even more discouraging, however, is the result that only 2 of 7 tests of antigen-antibody complexes using this antiserum gave positive results.

Even though the concepts of Litt has been confirmed by these results, the utility of the present technique as a useful assay is doubtful. Litt's previous work indicated a large amount of antibody was needed to give positive results. There is no hint in our own results that the smaller amounts of antigen-antibody complexes to be found in body fluids will give positive responses. In addition, the technique is very time consuming and positive results are obtained only 60% of the time.

Attempts to modify the in vitro technique for demonstration of polymorphonuclear cell chemotaxis, so that it can be used with eosinophils in an assay for antigen-antibody complexes have been unsuccessful to date.

Summary:

Using an antiserum containing a large amount of antibody, the eosinophilotactic properties of antigen-antibody complexes have been confirmed. The utility of the technique as an assay for complexes in body fluids is questioned. Attempts to develop a more sensitive in vitro technique have been unsuccessful.

Publications:

None .

LITERATURE CITED

1. Litt, M. 1960. Studies in experimental eosinophilia. I. Repeated quantitation of peritoneal eosinophilia in guinea pigs by a method of peritoneal lavage. Blood 16:1318-1329.
2. Litt, M. 1960. Studies in experimental eosinophilia. II. Induction of peritoneal eosinophilia by the transfer of tissues and tissue extracts. Blood 16:1330-1337.
3. Litt, M. 1961. Studies in experimental eosinophilia. III. The induction of peritoneal eosinophilia by the passive transfer of serum antibody. J. Immun. 87:522-529.

4. Litt, M. 1962. Studies in experimental eosinophilia. IV. Determinants of eosinophil localization. J. Allergy 33:532-543.
5. Litt, M. 1963. Studies in experimental eosinophilia. V. Eosinophils in lymph nodes of guinea pigs following primary antigenic stimulation. Amer. J. Path. 42:529-549.
6. Litt, M. 1964. Studies in experimental eosinophilia. VI. Uptake of immune complexes by eosinophils. J. Cell Biol. 23:355-361.
7. Litt, M. 1964. Studies in experimental eosinophilia. VII. Eosinophils in lymph nodes during the first 24 hr following primary antigenic stimulation. J. Immun. 93:807-813.

ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)
Task No. 1B662706A096 03: Laboratory Identification of Biological Agents
Work Unit No. 096 03 103: Rapid Diagnosis of Bacterial Infections by
Immunological and Chemical Means
Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland
Division: Bacteriology
Period Covered by Report: 1 July 1968 to 30 June 1969
Professional Author: Martha K. Ward, Captain, USPHS
Reports Control Symbol: RCS-MEDDH-288(R1)
Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OL0874	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8A. DES'N INSTR ^a	8B. SPECIFIC DATA- CONTRACTOR ACCESS	9. LEVEL OF SUM A. WORK UNIT
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES: ^a	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY	62706A	1B662706A096		03		103	
b. CONTRIBUTING	62124011	1B622401A096		03			
c. CONTRIBUTING	CDOG 1212b(9); 1412a(2)						
11. TITLE (Precede with Security Classification Code) ^a							
(U) Rapid diagnosis of bacterial infections by immunological and chemical means							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
003500 Clinical Medicine; 004900 Defense; 003200 BW, CW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
66 12		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: ^a				FISCAL		1	
c. TYPE:				YEAR		50	
d. AMOUNT:				CURRENT		1	
e. KIND OF AWARD:				70		50	
f. CUM. AMT.							
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a USA Medical Research Institute of Infectious Diseases				NAME: ^a Bacteriology Division			
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				Fort Detrick, Md 21701			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Crozier, D.				NAME: ^a Ward, M. K.			
TELEPHONE: 301 663-4111 Ext 5233				TELEPHONE: 301 663-4111 Ext 3246			
				SOCIAL SECURITY ACCOUNT NUMBER:			
21. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign intelligence considered.				NAME:			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Diagnosis; (U) Immunology; (U) Circulating antigen; (U) Antibodies;							
(U) Pneumococcus							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Develop more rapid, highly sensitive methods for specific early diagnosis of bacterial infections by detection of specific antigenic and metabolic products of the organism <u>in vivo</u> early in infection.							
24. (U) After producing appropriate antisera, attempt to detect circulating or tissue-fixed antigens early in infection by immunological and immunochemical techniques. Explore by chemical methods the possibility of detecting specific nonantigenic metabolic products.							
25. (U) 68 07 - 69 06 - Hemagglutination techniques using (1) antibody sensitized red blood cells for detection of small quantities of circulating pneumococcus antigen or (2) antigen sensitized cells for quantitation of antibodies from immunized animals have been developed. Technical difficulties with tanned cells have delayed studies on serial blood samples from infected animals for detection of circulating antigen. Factors affecting stability of tanned cells are being investigated.							

^aAvailable to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)
Task No. 1B662706A096 03: Laboratory Identification of Biological Agents
Work Unit No. 096 03 103: Rapid Diagnosis of Bacterial Infections by
Immunological and Chemical Means

Description:

Develop more rapid, highly sensitive methods for specific early diagnosis of bacterial infections by detection of specific antigenic and metabolic products of the organisms in vivo early in infection.

Progress:

Studies on early detection of circulating antigen, using pneumococcus infection as the model system, have been continued, but at a slowed pace, owing to personnel changes.

As reported last year,^{1/} a number of technical problems were encountered in studies using latex particles sensitized with antibodies in a hemagglutination (HA) test to detect small amounts of antigen. Results of tests were highly variable from one experiment to another and unaccounted-for, spontaneous agglutination frequently occurred in control tests.

In view of the above, a suitable substitute for latex particles has been sought. Direct sensitization of a variety of resin particles was examined and found unsatisfactory. Several chemical methods used by others for coupling of proteins to red blood cells (RBC) were examined and also found not to be useful in our system.

Although original trials to sensitize tanned sheep RBC with whole, hyperimmune antiserum had not been successful, the possible use of a tanned cell system was reexamined.

Hyperimmune antiserum was fractionated by 2 methods: (1) DEAE chromatography and (2) $(\text{NH}_4)_2\text{SO}_4$ precipitation. Although chromatography yielded a more nearly pure γ -globulin fraction than the crude precipitation method, attempts to sensitize tanned sheep cells with the DEAE fraction were not fruitful. The presence of the contaminants in the cruder fraction appeared to be necessary for best sensitization of tanned cells.

Titration of pneumococcus culture filtrates with tanned cells sensitized with 50% $(\text{NH}_4)_2\text{SO}_4$ fractions of antipneumococcus antiserum were found to be highly reproducible in preliminary studies. The tests were also very sensitive, detecting the presence of what was calculated to be nanogram quantities of polysaccharide.

Initial in vivo studies were very encouraging. Samples of plasma taken from injected animals as early as 12-16 hr after infection with approximately 200 pneumococci gave a positive HA test with antibody sensitized cells. The preinfection control samples from all animals were negative. The titer of the serial samples from test animals increased with time postinfection.

Unfortunately, before these critical in vivo tests could be extended and repeated, the HA test went completely out of control. All tanned cell preparations showed consistent, spontaneous agglutination.

Since there had been no change in batches of tannic acid, buffer system or other reagents, circumstantial evidence points to some change in the sheep cells. An investigation revealed that donor animals had been bled with unusual frequency recently. This may affect the stability of the cells. Another factor, that of the physiological changes associated with the estrous cycle of the female sheep, is also being investigated as a possible factor involved in the variation observed. Hopefully, the source of this variation will soon be pinpointed and controlled.

In other studies, we have developed a technique for sensitizing RBC with pneumococcus polysaccharide from crude culture filtrates. A HA test using antigen-sensitized cells has proved useful for titration of antibodies in serial bleedings from immunized animals, and appears to be more sensitive and more easily quantitated than the previously used quellung reaction and precipitin tests.

The possibility of using the HA inhibition test for detection of antigen is being explored.

Summary:

Hemagglutination techniques using (1) antibody sensitized RBC for detection of small quantities of circulating pneumococcus antigen or (2) antigen sensitized cells for quantitation of antibodies from immunized animals have been developed. Technical difficulties with tanned cells have delayed studies on serial blood samples from infected animals for detection of circulating antigen. Factors affecting stability of tanned cells are being investigated.

Publications:

None.

LITERATURE CITED

1. U. S. Army Medical Unit. 1 July 1968. Annual Progress Report, FY 1968, p. 219 to 223. Fort Detrick, Maryland.

ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)
Task No. 1B662706A096 03: Laboratory Identification of Biological Agents
Work Unit No. 096 03 104: Rapid Identification of Bacterial Agents by
Micro-methods
Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland
Division: Bacteriology
Period Covered by Report: 1 July 1968 to 30 June 1969
Professional Authors: Martha K. Ward, Captain, USPHS
Margaret L. Huff, M.P.H.
Reports Control Symbol: RCS-MEDDH-288(R1)
Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OLO882	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DISEN INSTR ^a	9. SPECIFIC DATA - CONTRACTOR ACCESS	10. LEVEL OF SUM
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES ^a	PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER
			1B662706A096		03		104
a. PRIMARY	62706A		1B622401A096		03		
b. Contracting	62124011						
c. Contracting	CDOG 1212b(9); 1412a(2)						
11. TITLE (Precede with Security Classification Code) ^a							
(U) Rapid identification of bacterial agents by micromethods							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
003500 Clinical Medicine; 004900 Defense; 003200 CW, BW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
66 12		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		80	
b. NUMBER: ^a				FISCAL		1	
c. TYPE: NA				YEAR		0	
d. AMOUNT:				CURRENT		0	
e. KIND OF AWARD:				70		0	
f. CUM. AMT.							
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a USA Medical Research Institute of Infectious Diseases				NAME: ^a Bacteriology Division			
ADDRESS: ^a Fort Detrick, Md 21701				ADDRESS: ^a USA Medical Research Institute of Infectious Diseases			
				Fort Detrick, Md 21701			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Crozier, D.				NAME: ^a Ward, M. K.			
TELEPHONE: 301 663-4111 Ext 5233				TELEPHONE: 301 663-4111 Ext 3246			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered.				ASSOCIATE INVESTIGATORS			
				NAME: Huff, M. L.			
				NAME: DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Rapid identification; (U) Microtechniques; (U) Fluorescent antibodies;							
(U) Replicate transfer							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Develop more rapid methods for isolation and specific identification of bacterial agents in samples collected by early warning air sampling devices. Employ these methods to examine clinical materials whenever applicable.							
24. (U) Reinvestigate the usefulness of microcultural methods for rapid identification of bacteria; explore the feasibility of identification of microcolonies in the living state; develop methods for replicate transfer of microcolonies for confirmatory studies.							
25. (U) 68 07 - 69 06 - The microculture and replicate "plating" technique previously described and used for rapid isolation and identification of a number of commonly encountered bacterial species (<u>Escherichia coli</u> , staphylococci, streptococci, etc.) has not proved useful for slower growing organisms such as <u>Pasteurella tularensis</u> , living vaccine strain. Of the more than 25 modifications of basal medium and technique, none yielded significant enhancement of growth of this organism in microculture.							
Work on this project has been temporarily discontinued.							

^a Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)
Task No. 1B662706A096 03: Laboratory Identification of Biological Agents
Work Unit No. 096 03 104: Rapid Identification of Bacterial Agents by
Micro-methods

Description:

Develop more rapid methods for isolation and specific identification of bacterial agents in samples collected by early warning air sampling devices. Employ these methods to examine clinical materials whenever applicable.

Progress:

A microculture procedure and a micro-modification of the standard replicate plating technique for rapid isolation and identification of certain bacterial species were described previously.^{1/} With the model system used, after 4-6 hr in microculture, replications of the growth could be transferred to a number of additional microculture slides or to clean slides (without growth medium) for fluorescent antibody and other staining techniques.

Unfortunately, certain pathogenic organisms of interest, e.g., Pasteurella tularensis, could not be grown rapidly in microculture; overnight or 24 hr incubation was required to obtain microcolonies on the media employed and under cultural conditions suitable for a number of other both gram negative and positive species.

Employing the vaccine strain of P. tularensis (LVS) as a model, studies during the current year have been directed toward attempts to modify the techniques developed in such a way as to stimulate growth from small inocula of this organism.

For the required microscopic examination of the microcultures, a blood-free medium is essential. The "T" medium of Tresselt and Ward was used as a basal medium for modification.^{2/}

A large variety of growth factors and other substances, known to affect bacterial metabolism and growth, were tested for their effect on time of appearance of colonies of LVS, both on the usual culture plates and in microculture. Conditions of incubation were also varied.

Despite a concentrated effort over the period of almost 6 months, no satisfactory solution to the problem was found. Of the more than 25 different substances studied in varying combinations and concentrations (including sterile "spent" medium recovered from liquid cultures in various phases of growth) only whole blood or blood products in anyway stimulated the growth of

small inocula. Even with blood, the 29-hr incubation period required for observable colonies was reduced by only 5-6 hr.

During the course of these studies, it was definitely shown that variations in batches of dehydrated products markedly affected the growth of this organism in the original basal medium. This observation has been called to the attention of the manufacturer.

In view of these very disappointing results, work on this project has been temporarily discontinued.

Summary:

The microculture and replicate "plating" technique previously described and used for rapid isolation and identification of a number of commonly encountered bacterial species (E. Coli, staph., strep., etc.) has not proved useful and slower growing organisms such as P. tularensis LVS strain. Of the more than 25 modifications of basal medium and technique none yielded significant enhancement of growth of this organism in microculture.

Work on this project has been temporarily discontinued.

Publications:

None.

LITERATURE CITED

1. U. S. Army Medical Unit. 1 July 1968. Annual Progress Report FY 1968. p. 225 to 228. Fort Detrick, Maryland.
2. Tresselt, H. B. and M. K. Ward. 1964. Blood-free medium for the rapid growth of Pasteurella tularensis. Appl. Microbiol. 12:504-507.

ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)
Task No. 1B662706A096 03: Laboratory Identification of Biological Agents
Work Unit No. 096 03 402: Development of Tests for Early Identification
of Viral Diseases
Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland
Division: Virology
Period Covered by Report: 1 July 1968 to 30 June 1969
Professional Author: Bruno J. Luscri, Ph.D.
Reports Control Symbol: RCS-MEDDH-288 (R1)
Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OL0855	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DES'N INSTR ^a	9. SPECIFIC DATA- CONTRACTOR ACCESS	9. LEVEL OF SUM
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY	62706A	1B662706A096		03		402	
b. CONTRIBUTING	62124011	1B622401A096		03			
c. CONTRIBUTING	CDOG 1212b(9)	1412a(2)					
11. TITLE (Precede with Security Classification Code) ^a							
(U) Development of tests for early identification of viral diseases							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
003500 Clinical Medicine; 004900 Defense; 003200 CW, BW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
61 08		CONT		DA		C. In-house	
17. CONTRACT/GRANT				19. RESOURCES ESTIMATE		4. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: ^a				FISCAL		2	
c. TYPE:				YEAR		30	
d. AMOUNT:				CURRENT		1	
e. CUM. AMT.				70		30	
18. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a USA Medical Research Institute of Infectious Diseases				NAME: ^a Virology Division			
ADDRESS: ^a Fort Detrick, Md 21701				ADDRESS: ^a USA Medical Research Institute of Infectious Diseases			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Crozier, D.				NAME: ^a Luscri, B. J.			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered.				ASSOCIATE INVESTIGATORS			
				NAME: DA			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Tissue culture; (U) Influenza; (U) Newcastle disease virus; (U) Vesicular stomatitis virus; (U) Antiviral activity; (U) Interferon							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) Develop tests for use in rapid diagnosis of viral diseases.							
24 (U) Produce an interferon of tissue culture origin. The interferon is to be studied for its antigenic properties, and attempts made to detect its presence by a suitable serological method employing specific antibody.							
25 (U) 68 07 - 69 06 - Soluble antiviral activities were not obtained when the continuous monkey kidney cell line LLC-MK-2 was exposed to live, or ultraviolet light treated influenza virus PR8, or Newcastle disease virus (NDV), designated AF (NDV-AF). Cumulative results suggest that this cell line may be defective in its capacity to synthesize an interferon.							
L cells (clone 929) from two sources (S1, S2) exposed to NDV, strain B1, yielded processed tissue culture fluids containing possible antiviral activity toward 2 strains of vesicular stomatitis virus (VSV) in a plaque-reduction bioassay. The bioassay procedure was not entirely satisfactory in one L cell source S2, since the challenge VSV was not consistently plaqued in maintenance medium (MM)-treated control L cell (S2) monolayers. L cell monolayers (S2) treated with high dilutions of the presumptive interferon containing fluids tended to reverse the inability of VSV to yield plaques in MM-treated L cell (S2) monolayers, in several bioassays.							
VSV plaqued satisfactorily in L cell (S1) monolayers, but this source failed to produce significant antiviral activity following exposure to NDV-AF.							
Attempts to improve the plaque-reduction bioassay procedure by employing selective L cell monolayers is planned, as well as attempts to produce and assay a neutralizing antibody for the putative interferon.							

^a Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)
Task No. 1B662706A096 03: Laboratory Identification of Biological Agents
Work Unit No. 096 03 402: Development of Tests for Early Identification
of Viral Diseases

Description:

Develop tests for use in the rapid diagnosis of viral diseases.

Progress:

Studies were continued in an attempt to develop a readily available source of an interferon for use in experiments to examine its inherent antigenicity.

Attempts to induce an interferon in LLC-MK₂ cell monolayers. In consecutive trials to examine possible interferon inducers in LLC-MK₂ cells, it was established that neither live, nor ultraviolet (UV) treated influenza virus, strain PR8, were capable of inducing antiviral activities against attenuated Venezuelan equine encephalitis (TC-83/3-3) or the Trinidad strain of Venezuelan equine encephalitis (VEE) when assayed by a cytopathic inhibition test. The cytopathic median tissue culture infectious dose (TCID₅₀) titers of TC-83/3-3, and of VEE were 10⁵ and 10⁶, respectively, when 0.2 ml of virus inoculum was used per tube culture of LLC-MK₂ cells.

Similarly, exposure of LLC-MK₂ cells to Newcastle disease virus (NDV), strain designation AF (NDV-AF) did not result in the production of virus-inhibiting substances as measured against a challenge with the New Jersey strain of vesicular stomatitis virus (VSV).

The results of these studies as well as previous attempts with attenuated VEE virus^{1/} suggest that the LLC-MK₂ cell line may be deficient or lacking in the capacity to produce interferon. Similar findings were made^{2/} with Vero cells, which did not produce interferon following exposure to either NDV, rubella, Sendai, or Sindbis viruses. It is probable, therefore, that coding for optimal interferon synthesis is uniquely dependent upon an intimate association between host cell and viral genome.

Some experimental evidence was obtained by observations which suggested that LLC-MK₂ cells may not be impaired in their ability to demonstrate interference towards attenuated VEE virus. It appeared that LLC-MK₂ cell monolayers surviving after exposure to either live or UV-treated influenza

virus PR8, and particularly following NDV-AF, were afforded some degree of protection subsequent to a challenge with attenuated VEE virus.

Production of a supposed interferon in L cell monolayers and some observations concerning its bioassay by the plaque-reduction method.

Cells. L cells (clone 929) from two sources grown as monolayer cultures were used. One line was supplied by the Physical Defense Division, Fort Detrick. These L cells were originally obtained from Microbiological Associates, Inc., (Bethesda, Md.) 9 months prior to our use and had been passaged every 5th day. These cells are designated L cells (S1).

Clone 929 L cells were obtained from the American Type Culture Collection (ATCC), and are designated L cells (S2).

Medium. The growth medium used principally was medium 199 and 10% fetal bovine serum (FBS). L cells (S2) were also grown in medium 199 with 15% calf serum (CS). The maintenance medium consisted of medium 199 with either 4% FBS, or 4% CS.

Tissue Culture. A confluent L cell (S1) monolayer was dispersed with 0.25% trypsin solution, centrifuged at room temperature, and the cells passaged to 5-75 cm² plastic tissue culture flasks (Falcon), or to 10-25 cm² plastic tissue culture flasks (Falcon).

L cells (S2) monolayers were generally dispersed with a 0.1% trypsin-versene solution in saline A, centrifuged at 4 C, and passaged to 5-75 cm² flasks or 10-25 cm² flasks. With an initial input of 10^{6.3} cells per flask, confluent cell sheets were obtained after incubation at 37 C in 3-4 days; at which time each culture contained approximately 10^{8.2} cells.

The 75 cm² flasks were generally used for exposure to Newcastle disease virus after 3-4 days, and the 25 cm² flasks were used in the plaque-reduction method for bioassay of putative interferon fluids after 24 hr at 37 C.

Viral stocks and titrations. Newcastle disease virus, was obtained from the VR Division, Fort Detrick. A designation for this strain is NDV-AF. The titer of NDV-AF was 10⁸ plaque forming units (PFU)/ml when assayed in chick embryo monolayers. NDV, strain B1 (NDV-B1) from the ATCC was propagated in 10-day-old embryonated eggs; allantoic fluids collected after incubation at 35 C for 48 hr were pooled, centrifuged at 1400 x g for 20 min, dispensed in small volumes, and placed at -56 C. The virus preparation titrated >10⁹ egg median lethal doses (ELD₅₀)/0.2 ml.

Vesicular stomatitis virus (VSV), New Jersey strain was obtained from VR Division, Fort Detrick. VSV, serotype Indiana, was supplied by the ATCC. Both VSV strains were propagated in chick embryo cell monolayers. VSV-New Jersey titrated 7.85 X 10⁶ PFU in L cells (S1), and VSV-Indiana yielded 1.3-1.8 X 10⁷ PFU/ml in L cells (S2).

Production of putative interferon. The growth medium was discarded, and each flask was washed once with Hanks' balanced salt solution (HBSS). After the HBSS was decanted from the cultures, each was inoculated with either 5×10^6 PFU NDV-AF, or $>10^8$ TCID₅₀ of NDV-B1. Control cultures received medium 199 solely. All cultures were incubated at 37 C for 60 min to allow virus adsorption. Excess inoculum was removed and each culture washed once with HBSS; then 10-ml quantities of medium 199 were added to each flask. All flasks were incubated at 37 C. The method is similar to that described by Youngner *et al.*^{3/}

Acid treatments of tissue culture fluids to eliminate NDV. In one method, the tissue culture fluids were harvested after 24 hr and adjusted to pH 2.0 by direct addition of concentrated HCl. After 6 days at 4 C, the fluids were adjusted to neutrality by direct addition of 1N or 5N NaOH. These fluids were stored frozen until used.

In another method, the tissue culture fluids were removed after 24 hr incubation and placed into dialysis units containing KCl-HCl buffer at pH 2.2. The units were held at 4 C for 6 days, after which the dialysis bags were transferred to sterile jars containing phosphate buffered saline (PBS), pH 7.4, and held at 4 C; the PBS was subsequently changed at 24 and 48 hr. Following dialysis the fluids were pooled, centrifuged at $210 \times g$ for 10 min, and dispensed into vials, for storage at -56 C. The resulting fluids were water clear.

Plaque-reduction assay for interferon.^{3,4/} L cell cultures were exposed for 20 hr to 3-ml 10-fold serial dilutions of the L-NDV processed fluids. Cultures of L cells were always overlaid with 3-ml amounts of MM for 20 hr, and occasionally with 3-ml amounts of L-control processed fluids. VSV in 0.2 ml amounts diluted in MM was added to the flasks. After 1 hr at 37 C, 5-ml amounts of an agar overlay consisting of double strength medium 199 plus final concentrations of 5% FBS, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 1% Nobles' agar was added to each flask. After 40 hr incubation at 37 C, 3-ml of a second agar overlay containing neutral red in a final concentration of 1:17,000 was added to each flask. Incubation was continued at 37 C for 4-5 hr when plaques were counted, and again in 1-2 days if plaques were not well defined earlier.

Protein determination. Protein was estimated with the Folin-Ciocalteu reagent, using bovine plasma albumin as a standard.^{5/}

Results of attempts to induce an interferon and the bioassay of such fluids on two sources of L cells.

L cells (S1). Cells from this source were grown in medium 199 + 10% FBS. In 4 experiments, the challenge virus, VSV-New Jersey was plaqued successfully in control monolayers treated with MM. An attempt at interferon production using NDV-AF as inducer did not yield processed tissue culture fluids (method 1) capable of inhibiting the challenge agent significantly. Hence, the New Jersey plaqued readily in MM-treated

monolayers but titration of L-NDV-AF processed fluids did not suggest a marked antiviral activity. The ability to plaque VSV-New Jersey in these cells treated with only MM was successful when 1, 3, 4, and 5-day-old cells were used.

L-cells (S2). Generally, the challenge agents VSV-New Jersey and Indiana, did not plaque in monolayers treated with only MM. This inconsistency of VSV to plaque in such control monolayers did not permit critical evaluation of the interferon content of L-NDV-B1 processed fluids which were examined. Cells grown in CS, or in medium 199 + 10% FBS and supplemented with 10% tryptose phosphate broth did not assist the plaquing of VSV in MM-treated monolayers. In one experiment, the absence of the penicillin-streptomycin mixture in the nutrient agar overlay appeared to increase the number of VSV plaques in the MM-treated cell cultures.

In 4 bioassays when VSV plaques were not obtained on MM-treated L cells, plaques did occur at high dilutions of the dialyzed L-NDV-B1 processed fluids (10^{-4} and 10^{-5}). These findings suggest that tissue culture fluids obtained from L cells exposed to NDV-B1 and treated to prepare a putative interferon possessed the capacity to reverse the apparent inability of VSV to yield definable plaques in MM-treated cell cultures.

Despite this difficulty, the results of 2 of the bioassays indicated that either S1 or S2 L cells exposed to NDV-B1 may yield processed fluids containing antiviral activity, suggestive of an interferon.

The L-NDV-B1 fluid prepared by dialysis was found to contain 50 μ g/ml of protein, whereas the L-control fluid contained no protein detectable by the Folin-Ciocalteu reagent.

Immunological studies. In an attempt to enhance their immune response guinea pigs were infected with attenuated VEE virus^{6/} 24 hrs prior to the intraperitoneal, then subcutaneous and footpad injections of the L-NDV-B1 fluids. Sera were obtained prior to, and following a reinjection of these fluids by the subcutaneous and footpad routes about 30 days after the initial injection of these fluids. An attempt to demonstrate a neutralizing antibody^{7/} for the putative interferon employing such sera is planned.

Summary:

A soluble antiviral activity was not demonstrable in processed tissue culture fluids following exposure of LLC-MK₂ cells to live influenza PR8, UV-treated influenza PR8, or Newcastle disease virus (NDV). The possibility that this cell line is deficient in the capacity to synthesize interferon was considered. The ability of LLC-MK₂ cells to demonstrate an interference phenomenon, however, may not be impaired.

L cells from two sources (S1, S2) were used in attempts to produce an L cell interferon inducible by 2 strains of NDV. Bioassay of the

putative interferons was performed by the plaque-reduction method using 2 strains of vesicular stomatitis virus (VSV) as the challenge agent.

L cells (S1) supported the plaquing of VSV-New Jersey in maintenance medium- (MM) treated L cell monolayers, but did not yield tissue culture fluids containing maximal antiviral activity after exposure to NDV-AF.

Processed tissue culture fluids following exposure of L cells (S1, S2) to NDV-B1 gave suggestive evidence of antiviral activity but the assays in general were not satisfactory due to the inconsistency of VSV to plaque in MM-treated L cell monolayers (S2). A common observation in several assays was that high dilutions of the putative interferon-containing solutions tended to reverse the inability of VSV to yield definable plaques on L cell (S2) monolayers. Attempts to improve the bioassay procedure by a selective source of L cells is planned.

Fluids possessing suggestive antiviral activity have been inoculated into guinea pigs previously injected with attenuated VEE in an attempt to provide an antiserum capable of neutralizing the putative interferon.

Publications:

None.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 03: Laboratory Identification of Biological Agents

Work Unit No. 096 03 403: Separation, Purification and Concentration of
Arbovirus Agents and Antigen-Antibody Complexes

Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland

Division: Virology

Period Covered by Report: 1 July 1968 to 30 June 1969

Professional Author: Neil H. Levitt, Captain, MSC

Reports Control Symbol: RCS-MEDDH-288 (R1)

Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OLO856	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUM ^a	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DISSEM INSTR ^a	9a. SPECIFIC DATA - CONTRACTOR ACCESS	9b. LEVEL OF SUM
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES: ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY	62706A	1B662706A096	03	403			
b. CONFIDENTIAL	62124011	1B622401A096	03				
c. CONFIDENTIAL	CDOG 1212b(9); 1412a(2)						
11. TITLE (Precede with Security Classification Code) (U) Separation, purification and concentration of arbovirus agents and antigen-antibody complexes							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a 003500 Clinical Medicine; 004900 Defense; 003200 BW, CW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
61 11		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		5	
b. NUMBER: ^a NA				FISCAL YEAR		1	
c. TYPE:				CURRENT		5	
d. AMOUNT:				70		1	
e. CUM. AMT.							
20. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a USA Medical Research Institute of Infectious Diseases				NAME: ^a Virology Division			
ADDRESS: ^a Fort Detrick, Md 21701				ADDRESS: ^a USA Medical Research Institute of Infectious Diseases			
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NAME: Crozier, D.				NAME: ^a Levitt, N. H.			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered.				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Arboviruses; (U) Immunology; (U) Antigens; (U) Antigen-antibody reactions; (U) Serology; (U) Tissue culture							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Study arbovirus agents in purified preparations, separate serological or immunological antigens from infectious virus particles, and separate group and specific antigens in order to investigate antigen-antibody complexes in concentrated and/or purified forms.							
24. (U) Selected group A arboviruses will be compared as to the infective and/or antigenic components. A cellulose column will be used for separation. Infectivity of each eluate will be determined using animals and cell cultures.							
25. (U) 68 07 - 69 06 - Personnel have recently been assigned. Studies are planned on viral sub-units, with evaluation of their antigenicity. These studies will be initiated in the near future when essential equipment is received.							

^aAvailable to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 03: Laboratory Identification of Biological Agents

Work Unit No. 096 03 403: Separation, Purification and Concentration of
Arbovirus Agents and Antigen-Antibody Complexes

Description:

Study arbovirus agents in purified preparations, separate serological or immunological antigens from infectious virus particles, and separate group and specific antigens in order to investigate antigen-antibody complexes in concentrated and/or purified forms.

Progress and Summary:

Personnel have recently been assigned. Studies are planned on viral sub-units with evaluation of antigenicity. These studies will be initiated in the near future when essential equipment is received.

Publications:

None.

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ANNUAL PROGRESS REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)
Task No. 1B662706A096 03: Laboratory Identification of Biological Agents
Work Unit No. 096 03 800: Immunological Studies with Microbial Toxins
Reporting Installation: U. S. Army Medical Research Institute of
Infectious Diseases
Fort Detrick, Maryland
Division: Bacteriology and Animal Assessment
Period Covered by Report: 1 July 1968 to 30 June 1969
Professional Authors: Virginia G. McGann, Ph.D.
Charles H. Hobbs, Captain, VC
Elizabeth O. Roberts, Ph.D.
Reports Control Symbol: RCS-MEDDH-288(R1)
Security Classification: UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OLO876	69 07 01	DD-R&E (AR) 636	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DISSEM INSTR ^a	9. SPECIFIC DATA- CONTRACTOR ACCESS	9. LEVEL OF SUM
68 07 01	D. CHANGE	U	U	NA	DE	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES: ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62706A		1B662706A096		03	
b. Contributing		62124011		1B622401A096		03	
c. Contributing		CDOG 1212b(9)		1412a(2)		800	
11. TITLE (Precede with Security Classification Code) ^a							
(U) Immunological studies with microbial toxins							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
003500 Clinical Medicine; 004900 Defense; 003200 CW, BW, RW							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
66 10		CONT		DA		C. In-house	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE:				PRECEDING		b. FUNDS (in thousands)	
b. NUMBER: ^a				69		3	
c. TYPE:				FISCAL YEAR		50	
NA				CURRENT		50	
d. AMOUNT:				70		3	
e. KIND OF AWARD:				f. CUM. AMT.			
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: ^a USA Medical Research Institute of Infectious Diseases				NAME: ^a Bacteriology Division			
ADDRESS: ^a Fort Detrick, Md 21701				ADDRESS: ^a USA Medical Research Institute of Infectious Diseases			
				Fort Detrick, Md 21701			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: Crozier, D.				NAME: ^a McGann, V. G.			
TELEPHONE: 301 663-4111 Ext 5233				TELEPHONE: 301 663-4111 Ext 6130			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence considered.				ASSOCIATE INVESTIGATORS			
				NAME: Hobbs, C. H.			
				NAME: Roberts, E. O.			
				DA			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Enterotoxin, staphylococcal; (U) Immunology; (U) Immuno-electrophoresis; (U) Toxoid; (U) Antibodies							
23. TECHNICAL OBJECTIVE, ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23 (U) Investigate immunologic responses of a susceptible host after exposure to microbial toxin and after immunization with toxoid.							
24 (U) Evaluate <u>in vitro</u> methods to determine protective status of antibodies in a susceptible host and to assay for immunogenicity of toxoid preparations. Investigate suitable techniques to compare antigenic composition of a variety of preparations of enterotoxins.							
25 (U) 68 07 - 69 06 - Immunological studies with staphylococcal enterotoxin B (SEB) have continued. Vaccinated monkeys that received the initial lot of formalin-treated SEB toxoid showed no significant decrease in circulating antibody or in protection against challenge throughout the course of 1 year. The toxoid maintained immunogenicity for at least 18 months when stored at 4 C. Physical, electrophoretic, serologic and immunogenic properties of a new production lot of toxoid were essentially the same as those of the initial lot.							
In monkeys immunized with toxoid, hemagglutinin titers were not a satisfactory index of protection against SEB. Toxin precipitins, regardless of titer, and high-titering toxoid precipitins were clearly associated with protection.							
Toxin given by intravenous route, but not by the respiratory route, was considerably more effective than toxoid in stimulating antibody response. Potentiation of the activity of toxoid by altering dosage or immunization schedule, by addition of adjuvant, or by combination with other vaccines is under investigation.							
Studies were initiated to investigate techniques for detecting SEB in biologic fluids and for comparing antigenic activity of a variety of preparations of enterotoxins A and B.							

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B622706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 03: Laboratory Identification of Biological Agents

Work Unit No. 096 03 800: Immunological Studies with Microbial Toxins

Description:

Investigate immunologic response of a susceptible host after exposure to microbial toxin and after immunization with toxoid.

Progress:

Studies have continued on evaluation of in vitro methods for measuring the efficacy of immunization with formalin-treated toxoids of staphylococcal enterotoxin B (SEB). Preliminary investigations of methods for detecting SEB in biologic fluids after exposure and for comparing a variety of enterotoxin preparations are in progress.

Formalin-treated toxoid: In an earlier report^{1/} safety, efficacy and antibody response to a small lot of toxoid (Lot 44) were described. Studies to evaluate duration of immunity in vaccines and shelf-life of toxoid were in progress. Groups of monkeys vaccinated with 2 doses of 80 µg N in a 4-week interval were challenged with SEB at 6 weeks, 6 months and 1 year after vaccination. Protection against challenge was equally effective for all groups, and antibody titrations on serial bleedings indicated that hemagglutinin titers for SEB coupled to erythrocytes with bis-diazotized benzidine (BDB-HA) and toxoid-precipitin titers were maintained for at least 1 year. Toxin-precipitin titers were variable, but in animals challenged at 1 year the prechallenge titers were the same or greater than the titers at 6 week post-vaccination. As in previous studies, protection against lethality was associated with the presence of toxin-precipitating antibody. Toxoid stored for 18 months at 4 C was as effective as fresh toxoid in stimulating antibody production and in protection against challenge. Ultracentrifuge analysis of stored toxoid (by Dr. Anne Buzzell, Physical Sciences Division, USAMRIID) indicated that large, loose aggregates of toxoid formed during storage, but readily broke apart to yield a predominately dimer population.

A production lot of toxoid (Lot 87285) was received from Chas. Pfizer & Co., Inc. (Contract No. DADA 17-68-C-8079) in July 1968. Physical, electrophoretic and serologic properties were essentially the same as those determined previously for freshly prepared Lot 44. Detailed description of safety testing and biologic response of immunized monkeys is reported under Work Unit No. 096 02 002, Part VI.

Analysis of serologic response and its relationship to protection is in progress. Studies have been completed on groups of monkeys that were immunized

subcutaneously with 2 doses of 50 μ g N (312 μ g protein), given at a 4-week interval. Antibody response to immunization (Table I) was similar to the response to Lot 44. After a single injection essentially all animals produced BDB-HA and toxoid-precipitating antibody, but only 25% produced toxin-precipitins. After a second injection, all titers increased and approximately 70% of the animals had toxin-precipitins.

TABLE I. ANTIBODY RESPONSE OF MONKEYS TO IMMUNIZATION WITH SEB TOXOID, LOT 87285 (36/GROUP).

ANTIBODY TITRATION		RESPONSE TO IMMUNIZATION AFTER INITIAL INJECTION ^{a/}				
		0 Week	2 Week	4 Week	7 Week	10 Week
BDB-HA	No. positive	0	31	35	36	35
	Median titer	Neg	1:40	1:80	1:320	1:320
Toxoid Precipitin	No. positive	0	32	29	35	32
	Median titer	Neg	1:2	1:2	1:8	1:4
Toxin Precipitin	No. positive	0	4	10	20	25
	Median titer	Neg	Neg	Neg	1:1	1:1

^{a/} Immunization: 2 doses, 50 μ g N/dose. 1st injection, after 0-week bleeding; 2nd injection, after 4-week bleeding.

After the 10-week bleeding, animals were challenged with SEB at doses of 10 or 300 μ g/kg. Response to challenge as compared with prechallenge antibody titer is shown in Table II. BDB-HA titers were not a satisfactory index for resistance of immunized monkeys to SEB; titers of animals responding to challenge ranged from 1:80-1:640, whereas titers of non-responders ranged from 1:80-1:5120. Protection was associated with precipitating antibody. The relative contribution of toxoid- and toxin-precipitins was difficult to evaluate because toxoid-precipitins were present in all animals that had toxin-precipitins. One monkey, however, that survived challenge with the 300 μ g dose of SEB had only toxoid-precipitins. In general, magnitude of titer was an important factor in assessing the significance of toxoid-precipitins, but not of toxin-precipitins. Animals with toxin-precipitating antibody, regardless of titer, showed no overt response to challenge with 10 μ g/kg and survived challenge with 300 μ g/kg.

TABLE II. ANTIBODY TITER AND OVERT RESPONSE OF IMMUNIZED MONKEYS TO CHALLENGE WITH SEB.

CHALLENGE DOSE (μ g/kg)	RESPONSE TO CHALLENGE					
	BDB-HA		Toxoid Precipitin		Toxin Precipitin	
	Prechallenge Titer	$\frac{R^a}{T}$	Prechallenge Titer	$\frac{R}{T}$	Prechallenge Titer	$\frac{R}{T}$
10	1:80	1/2	Neg	2/2	Neg	4/6
	1:160	1/3	+ <u>b/</u>	1/2	+	0/4
	1:320	1/5	1:1	1/1	1:1	0/5
	1:640	1/4	1:2	0/4	1:2	0/1
	> 1:640	0/3	> 1:2	0/8	> 1:2	0/1
300	1:80	0/1	Neg		Neg	3/4
	1:160	1/3	+	0/1	+	0/2
	1:320	2/6	1:1	1/1	1:1	0/5
	1:640	0/4	1:2	2/3	1:2	0/5
	> 1:640	0/2	> 1:2	0/8	> 1:2	0/1

$$\frac{a}{\frac{R}{T}} = \text{At } 10 \mu\text{g/kg} = \frac{\text{Number ill}}{\text{Number tested}}; \text{ at } 300 \mu\text{g/kg} = \frac{\text{Number dead}}{\text{Number tested}}$$

b/ + = Detectable precipitin but too weak for titration

Toxoid was considerably less effective than toxin in evoking antibody response in monkeys. Table III summarizes the responses of monkeys after challenge with SEB at 10 μ g/kg by the intravenous (IV) and respiratory routes. The animals were nonimmunized, survivor controls from toxoid evaluation studies. No toxoid-precipitating antibody was produced by a single exposure to SEB, but other studies indicated that these antibodies were found in approximately 50% of animals after several exposures to higher doses. BDB-HA and toxin-precipitin responses to SEB at 10 μ g/kg by the IV route were essentially equivalent to those following immunization with 2 doses of 50 μ g toxoid N/dose. Challenge by the respiratory route was much less effective in stimulating antibody production.

TABLE III. ANTIBODY RESPONSE OF MONKEYS AFTER IV AND RESPIRATORY CHALLENGE WITH SEB, 10 $\mu\text{g/kg}$.

CHALLENGE ROUTE	ANTIBODY TEST	RESPONSE TO CHALLENGE			
		2 Week		6 Week	
		Pos. Total	Median titer	Pos. Total	Median titer
IV	BDB-HA	21/35	1:10	34/35	1:320
	Toxoid Precipitin	0/35	Neg	0/35	Neg
	Toxin Precipitin	16/35	Neg	19/35	+a/
Respiratory	BDB-HA	5/18	Neg	10/18	1:20
	Toxoid Precipitin	0/18	Neg	0/18	Neg
	Toxin Precipitin	1/18	Neg	4/18	Neg

a/ + = Detectable precipitin but too weak for titration

Attempts to potentiate antibody response to toxoid are in progress. Simultaneous immunization with toxoid and typhoid + paratyphoid A and B (TAB) vaccine or living tularemia vaccine gave the same response as toxoid alone. Studies on dosage and immunization schedules are not yet completed. Preliminary results indicate that time of appearance, incidence and magnitude of antibody response are dose related. A single injection of low doses of toxoid ($<80 \mu\text{g N}$) produced BDB-HA and toxoid-precipitating antibody within 2 weeks, but 2 injections were usually required for production of toxin-precipitins. At higher doses, toxoid-precipitins appeared before hemagglutinins, and toxin-precipitins developed in 50% of the animals within 4 weeks. Combination of toxoid with adjuvant is under investigation at Chas. Pfizer & Co., Inc. (Contract No. DADA 17-68-C-8079).

Analysis and Detection of Enterotoxin. Studies were initiated to investigate techniques for detecting SEB in biologic fluids and for comparing antigenic activity of various preparations of enterotoxins A and B.

HA inhibition, double diffusion and single diffusion in agar gel were equally sensitive for detecting SEB in biologic fluids; 0.001-0.005 μg could

be detected in monkey or rabbit plasma. Time of appearance and duration of circulating SEB appeared to be related to dose, route and outcome of challenge. Circulating SEB was present from 15 min-5 hr after challenge of one monkey that survived a dose of 400 μ g/kg; in another monkey that succumbed, no circulating SEB was found after 1 hr.

Procedures for comparison of crude, partially purified, conjugated or polymerized enterotoxin are being evaluated. Survey of available sera indicated that hyperimmunization with purified SEB, Lots 14-30 or 14-31 R, stimulated production of 4-5 antibodies reacting with antigens other than SEB. These antibodies appeared only after multiple exposures to high concentrations of SEB. Procedures are not yet available for identifying the antigens that may be responsible.

Summary:

Vaccinated monkeys that received the initial lot of formalin-treated SEB toxoid showed no significant decrease in circulating antibody or in protection against challenge throughout the course of 1 yr. The toxoid maintained immunogenicity for at least 18 months when stored at 4 C. Physical, electrophoretic, serologic and immunogenic properties of a new production lot of toxoid were essentially the same as those of the initial lot.

In monkeys immunized with toxoid, BDB-HA titers were not a satisfactory index of protection against SEB. Toxin precipitins, regardless of titer, and high-titering toxoid precipitins were clearly associated with protection.

Toxin injected intravenously was considerably more effective than toxoid in stimulating antibody response, but respiratory exposure to toxin was less effective. Potentiation of the activity of toxoid by altering dosage or immunization schedule, by addition of adjuvant, or by combination with other vaccines is under investigation.

Studies were initiated to investigate techniques for detecting SEB in biologic fluids and for comparing antigenic activity of a variety of preparations of enterotoxins A and B.

Publications:

None.

LITERATURE CITED

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APPENDIX A

U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
GUEST LECTURE SERIES

<u>DATE</u>	<u>GUEST LECTURER</u>	<u>TITLE OF PRESENTATION</u>
26 Sep 68	Captain Elgin C. Cowart, Jr., MC, USN Curator of the Medical Museum Armed Forces Institute of Pathology Washington, D. C.	A Historical Review of the Development of the Armed Forces Medical Museum.
10 Oct 68	Dr. Aaron D. Alexander Chief, Veterinary Microbiology Div Walter Reed Army Institute of Research Washington, D. C.	Leptospirosis and Melioidosis.
21 Nov 68	Lt Colonel Robert J. T. Joy, MC Chief, Medical Research Div U. S. Army Medical Research and Development Command Washington, D. C.	Application of Physiology to Military Problems.
12 Dec 68	Colonel Robert Altman, MSC Executive Secretary, Armed Forces Pest Control Board Washington, D. C.	Highlights of Medical Entomology for 1968.
6 Feb 69	Dr. Harry M. Meyer, Jr. Chief, Laboratory of Viral Immunology Division of Biologics Standards National Institutes of Health Bethesda, Maryland	The Rubella Vaccine Story.
13 Mar 69	Dr. Ronald A. Ward Department of Entomology Walter Reed Army Institute of Research Washington, D. C.	African Trypanosomiasis.
10 Apr 69	Dr. Kevin Barry Chief, Department of Renal Research and Director of Medical Education Washington Hospital Center Washington, D. C.	Prevention and Treatment of Acute Renal Failure.
8 May 69	Captain R. C. Atkinson, USN U. S. Naval Oceanographic Office Washington, D. C.	An Overview of Marine Sciences

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APPENDIX B

U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
PROFESSIONAL STAFF MEETINGS

<u>DATE</u>	<u>LECTURER</u>	<u>TITLE OF PRESENTATION</u>
20 Sep 68	Captain Robert S. Pekarek, MSC Physical Sciences Division	Infection-related Acute Changes in Trace Elements in Serum.
	Captain David A. Rhoda, VC Physical Sciences Division	Influences of Staphylococcal Enterotoxin B upon Body Fluid Compartmentalization.
	Captain David Auerbach, VC Physical Sciences Division	Influences of Staphylococcal Enterotoxin B upon Coagulation Homeostasis.
18 Oct 68	Captain Martha K. Ward, USPHS Chief, Bacteriology Division	Introductory Remarks of Program to be Presented.
	Captain Charles P. Craig, MC Medical Division	Discussion of Problems in Inter- pretation of Immunological Data.
	Captain Paul A. Klein, MSC Bacteriology Division	Some Observations on a Combina- tion Live Vaccine in Mice.
	Dr. Virginia G. McGann Bacteriology Division	Significance of Serologic Reactions.
	Mrs. Mary H. Wilkie Bacteriology Division	Evaluation of the Immune Response.
15 Nov 68	Lt Colonel Robert W. McKinney, MSC Chief, Virology Division	Slides on Viet Nam and Saigon were presented.
	Major Emerson L. Shroyer, MSC Virology Division	The Spotted Fever Group of Rickettsiae
	Miss Marie L. Miesse Virology Division	The Rickettsiae.
17 Jan 69	Lt Colonel Peter J. Bartelloni, MC Chief, Medical Division	Behavioral Effects of Infectious Disease.
		Clinical Evaluation of Eastern and Western Equine Encephalitis Vaccines.

<u>DATE</u>	<u>LECTURER</u>	<u>TITLE OF PRESENTATION</u>
17 Jan 69 (Cont'd)	Captain Frank M. Calia, MC Medical Division	Absorption and Excretion of Chloromycetin and Amphotericin in Humans.
	Captain John L. Winnacker, MC Medical Division	Growth Hormone Responses during Infection.
28 Feb 69	Captain Ronald E. Vaughn, VC Animal Assessment Division	Relationship of TC-83 Vaccination of Pregnant Mice to Litter Size and Survival of the Neonates.
	Captain Frank E. Chapple, VC Animal Assessment Division	Cross-circulation Therapy in Yellow Fever-infected Rhesus Monkeys.
	Captain Charles H. Hobbs, VC Animal Assessment Division	The Efficacy and Safety of Staphylococcal Enterotoxin B.
	Mr. Ralph W. Kuehne Animal Assessment Division	The Efficacy of Single and Combined Arbovirus Vaccines by Various Routes of Administration.
21 Mar 69	Major William S. Collins, II, MSC Pathology Division	Instrumentation for the Separation and Assay of Proteins.
	Major Hubert J. Wolfe, MC Pathology Division	Enzyme, Isotope, and Fluorescein Markers in the Study of the Pharmacology of Staphylococcal Enterotoxin B.
	Colonel Joseph F. Metzger, MC Chief, Pathology Division	Methods to Increase Antigenicity of Staphylococcal Enterotoxin B.
18 Apr 69	Mr. Charles O. Roberts Microbiology Division	Serological Studies of <u>Pasteurella pestis</u> .
	Mr. Daniel N. Harrison Microbiology Division	Unity of <u>Pasteurella pestis</u> during a Plague Epidemic.
	Lt Colonel John D. Marshall, Jr., MSC Chief, Microbiology Division	Status of the Attenuated Plague Vaccine.
		Fibrinolysin - Controlling factor in the Epidemiology of Plague?

<u>DATE</u>	<u>LECTURER</u>	<u>TITLE OF PRESENTATION</u>
23 May 69	Captain Martha K. Ward, USPHS, Chairman Chief, Bacteriology Division	An Inter-division Seminar on Combined Vaccines.
	Lt Colonel Peter J. Bartelloni, MC Chief, Medical Division	
	Lt Colonel John D. Marshall, Jr., MSC Chief, Microbiology Division	
	Lt Colonel Robert W. McKinney, MSC Chief, Virology Division	
	Major Richard O. Spertzel, VC Chief, Animal Assessment Division	
	Dr. Francis E. Cole, Jr. Virology Division	

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APPENDIX C

U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
FORMAL PRESENTATIONS AND BRIEFINGS

<u>Date and Group or Individual</u>	<u>Individual(s) Participating</u>	<u>Subject</u>
5 Jul 68 Global Medicine Course Walter Reed Army Institute of Research	Lt Colonel John D. Marshall, Jr., MSC	Plague.
18 Jul 68 Department of Pathology Seminar University of Connecticut Storrs, Connecticut	Lt Colonel Gilberto S. Trevino, VC	Pathology of Snake Venoms.
25 Jul 68 Dr. Russell D. O'Neal Asst Secretary of the Army (R&D)	Colonel Dan Crozier, MC	Medical Defense Aspects of BW
12-13 Aug 68 Medical Advisory Committee Deseret Test Center Fort Douglas, Utah	Colonel Dan Crozier, MC	Medical Problems of BW.
28 Aug 68 Colonels William B. O'Neill, Anthony Urgine, George L. Robertson; Major J. S. Kennan; Mr. Harry S. Lucia, Medical Intelligence Ofc, OTSG	Colonel Nicholas F. Conte, MC Major Robert A. Massey, MSC	Medical Intelligence Requirements of BW Defense.

<u>Date and Group or Individual</u>	<u>Individual(s) Participating</u>	<u>Subject</u>
5 Sep 68 Commission on Epidemiological Survey, Armed Forces Epidemiolog- ical Board	Colonel Dan Crozier, MC Colonel William R. Beisel, MC Lt Colonel Robert W. McKinney, MSC Captain Charles P. Craig, MC	<u>Medical Defense Against BW Introductory Remarks.</u> Metabolic Approach to Diagnosis. Immunoprophylaxis Research Program. Research Program in Rapid Diagnosis.
6-16 Sep 68 8th International Congress of Tropical Medicine and Malaria Tehran, Iran	Lt Colonel John D. Marshall, Jr., MSC	Survey of Respiratory Infection Among Persons exposed to <u>P. pestis: The Gradation of Clinical Symptoms.</u>
11 Sep 68 Aeromedical-Biosciences Panel of USAF Scientific Advisory Board, HQ, USAF, Washington, D. C.	Colonel Dan Crozier, MC	Medical Defensive Aspects of BW.
26 Sep 68 Dr. Herbert Pollock Institute of Defense Analysis	Colonel Dan Crozier, MC Colonel Nicholas F. Conte, MC Colonel William R. Beisel, MC	Metabolic Mechanisms of Infection.
4-5 Oct 68 Symposium on Surgical Sepsis, State University of New York Buffalo, New York	Colonel William R. Beisel, MC	Experimental Human Infection and Hyperthermia: Metabolic Aspects.
7 Oct 68 Chiefs of Professional Divisions, Dept of Medicine, Ohio State University, Columbus, Ohio	Colonel William R. Beisel, MC	Research Approaches in Infectious Disease.

<u>Date and Group or Individual</u>	<u>Individual(s) Participating</u>	<u>Subject</u>
8 Oct 68 Division of Infectious Diseases Seminar, Ohio State University Columbus, Ohio	Colonel William R. Beisel, MC	Hepatic Enzyme Responses to Induced Infection.
8-10 Oct 68 Public Health Service Advisory Committee on Immunization Practices, National Communicable Disease Center, Atlanta, Georgia	Colonel Dan Crozier, MC	Report on Current Development and Use of Encephalitis Vaccines.
15 Oct 68 Biochemistry Group Walter Reed Army Institute of Research	Colonel William R. Beisel, MC	Guest Lecturer. Title: Factors Controlling Amino Acid Metabolism.
17-18-19 Oct 68 Commission on Immunization, Armed Forces Epidemiological Board	Colonel Dan Crozier, MC	Present Status of Vaccines of BW Interest.
5 Nov 68 Department of Defense Personnel (FSTC Group)	Colonel Nicholas F. Conte, MC	Medical Defense Aspects of BW.
7 Nov 68 Global Medicine Course Walter Reed Army Institute of Research	Colonel William R. Beisel, MC Captain Charles P. Craig, MC Captain Robert S. Pekarek, MSG Captain John L. Winnacker, MC	<u>Early Effects of Infectious Disease Metabolic Aspects of Host Response.</u> Physical and Serological Diag- nostic Techniques. Trace Element Changes in Infection. Endocrine Aspects of Host Response.

SubjectIndividual(s)
ParticipatingDate and
Group or Individual

The Biological Warfare Problem.

Colonel Dan Crozier, MC

11 Nov 68

96th Annual Meeting of the
American Public Health Assn.,
Detroit, Michigan

Guest Lecturer in Biology Section.
Title: Genetic and Embryologic
Aspects of Growth.

Colonel William R. Beisel, MC

11-12 Nov 68

Frederick Community College
Frederick, Maryland

Orientation Briefing and Tour of
the New Medical Facility.

Colonel Dan Crozier, MC
Lt Colonel Gilberto S. Trevino, VC

20 Nov 68

Brigadier General Wilson M.
Osteen, VC, Chief, Veterinary
Corps, OTSG

Introduction to Medical Problem.

Colonel Dan Crozier, MC

21 Nov 68

Senior Navy Medical Professional
Personnel

Immunophylaxis.

Lt Colonel Robert W. McKinney, MSC

Diagnosis and Identification.

Captain Charles P. Craig, MC

Metabolic Response of the Host.

Colonel William R. Beisel, MC

Pathophysiological Responses to
Staphylococcal Enterotoxin B (SEB).

Colonel William R. Beisel, MC

22 Nov 68
Munitions Command Advisory
Committee: Dr. Walter J. Nungester,
Chairman; Drs. Paul M. Gross,
Jake T. Nolen, and John A. Zapp.

Immunological Responses to SEB.

Dr. Virginia G. McGann

Production and Evaluation of EEE
Vaccine.

Dr. Francis E. Cole, Jr.

Immune Responses during Attenuated
VEE Infection.

Captain Charles P. Craig, MC

<u>Date and Group or Individual</u>	<u>Individual(s) Participating</u>	<u>Subject</u>
22 Nov 68 Munitions Command Advisory Committee (Cont'd)	Captain Robert S. Pekarek, MSC	Trace Element Changes during Acute Infection.
	Lt Colonel Peter J. Bartelloni, MC	Influence of Acute Infection on Human Performance.
26 Nov 68 Foreign Exchange Students: Major Dr. Johann Sailer, VC, West German Ministry of Defense, Bonn; Major Dr. Rudiger Vierling, VC, West German Ministry of Defense, Bonn; Dr. Harold von Sprockhoff, West German Armed Forces Testing Sta.	Colonel Nicholas F. Conte, MC Colonel William R. Beisel, MC Dr. Virginia G. McGann Lt Colonel Peter J. Bartelloni, MC	Welcome and Introduction. Pathophysiologic Responses to SEB. Immunologic Responses to SEB. Clinical Features Following Accidental Exposure to SEB.
1-4 Dec 68 5th National Meeting of Reticuloendothelial Society, New York, New York	Captain Charles P. Craig, MC Captain Edward V. Staab, MC	Effect of Attenuated VEE Virus Infection on Immunity. Reticuloendothelial System Function in Guinea Pigs Infected with Attenuated VEE Virus.
5-6 Dec 68 Commission on Rickettsial Diseases, Armed Forces Epidemiological Board	Colonel Dan Crozier, MC	Present Status of Q Fever and Rocky Mountain Spotted Fever Vaccines.
19 Dec 68 Colonel George Snead, Jr. (BG designate) Director of Army Research	Colonel Dan Crozier, MC	Orientation Briefing and tour of the new Medical Facility.

<u>Date and Group or Individual</u>	<u>Individual(s) Participating</u>	<u>Subject</u>
10 Jan 69 Department of Defense Personnel (FSTC Group)	Colonel Nicholas F. Conte, MC	Medical Defense Aspects of BW.
15 Jan 69 Military Medical and Allied Sciences Basic Science Class, Walter Reed Army Institute of Research	Dr. William R. Beisel	Endocrine Changes during Infection.
22 Jan 69 Briefing for Brigadier General James A. Hebbeler, Director of CBR&N Operations, O/ACSFOR	Lt Colonel Peter J. Bartelloni, MC	Biological-Medical Effects, SEB.
28 Jan 69 Walter Reed Hospital Dietitians Walter Reed General Hospital	Dr. William R. Beisel	Changing Concepts in Diabetes.
31 Jan 69 Mr. Albert E. Hayward, Colonel Richard H. Taylor, OSD, DDR&E	Colonel Dan Crozier, MC	General Briefing.
13-14 Feb 69 Armed Forces Epidemiological Board Meeting	Colonel Dan Crozier, MC	Presentation of Report of the Commission on Epidemiological Survey to the Board.
17-18 Feb 69 Army Science Advisory Panel Edgewood Arsenal, Maryland	Colonel Dan Crozier, MC	Medical Defense Against BW.
26 Feb 69 Global Medicine Course Walter Reed Army Institute of Research	Lt Colonel John D. Marshall, Jr., MSC	Plague.

<u>Date and Group or Individual</u>	<u>Individual(s) Participating</u>	<u>Subject</u>
12 Mar 69 Walkersville High School Walkersville, Maryland	Major John L. Winnacker, MC	The Endocrine System.
12 Mar 69 Maryland Branch of the American Society of Microbiology, Baltimore, Maryland	Captain Martha K. Ward, USPHS	Studies on Combined Antigens.
19 Mar 69 Pre-Medical Society The University of Maryland School of Medicine, Baltimore, Maryland	Dr. William R. Beisel	A Future in Medical Research.
19 Mar 69 Life Sciences Research Program Army Research Office Washington, D. C.	Colonel Dan Crozier, MC Colonel Nicholas F. Conte, MC Dr. William R. Beisel Lt Colonel Robert W. McKinney, MSC Major Robert A. Massey, MSC Mr. J. R. Everly	To present the annual technical and budgetary review.
20 Mar 69 Naval Medical School, Naval Medical Center, Bethesda, Md.	Colonel Dan Crozier, MC	Medical Defense Aspects of BW.
21 Mar 69 LTC Ralph-Ulrick H. Meyer Asst Director of Medical Services, Eastern Command, Royal Australian Army Medical Corps	Colonel Nicholas F. Conte, MC	General briefing.
24 Mar 69 Dr. Francis Gordon, Dr. Shepler National Academy of Sciences	Dr. William R. Beisel	Early Diagnosis of Infection in Astronauts.

<u>Date and Group or Individual</u>	<u>Individual(s) Participating</u>	<u>Subject</u>
24 Mar 69 Dr. Joseph C. Olson, Dr. Ezra Casman, USPHS, Food and Drug Administration	Dr. William R. Beisel Colonel Nicholas F. Conte, MC LTC Peter J. Bartelloni, MC Dr. Virginia G. McGann	Briefing on SEB Studies.
24 Mar 69 Department of Microbiology Guest Lecture Program Howard University, Washington, D.C.	Lt Colonel John D. Marshall, Jr.	Plague and Tularemia.
3 Apr 69 Military Nursing Practice and Research Course Students, Walter Reed Army Institute of Nursing	Colonel Nicholas F. Conte, MC Lt Colonel Jane Johnston, ANC Captain David A. Rhoda, VC	General briefing and tour of the new medical facility.
9 Apr 69 Walkersville High School Walkersville, Maryland	Dr. William R. Beisel	Defending Man Against BW.
17 Apr 69 Federation of American Societies for Experimental Biology, Atlantic City, New Jersey	Dr. Robert S. Pekarek	Trace Metal Metabolism during Infection and Endotoxemia.
19 Apr 69 Maryland Chapter, American Society of Microbiology, Ft Detrick, Md.	Captain Michael C. Powanda, MSC Dr. Robert S. Pekarek	Tryptophan and Typhimurium. Trace Metal Metabolism during Infection and Endotoxemia.
25 Apr 69 Endocrine-Metabolic Conference Walter Reed General Hospital	Major Kenneth A. Woeber, MC	Thyroid.

<u>Date and Group or Individual</u>	<u>Individual(s) Participating</u>	<u>Subject</u>
7 May 69 Dietetic Administration Course Walter Reed Army Institute of Research	Dr. William R. Beisel	Another Look at Diabetes Mellitus.
15 May 69 Dr. G.E. Gordon Smith and Prof. David G. Evans, Microbiological Research Estab- lishment of United Kingdom	Colonel Dan Crozier, MC Dr. William R. Beisel	Medical Defense Against BW.
16-17 May 69 Armed Forces Epidemiological Board Meeting	Colonel Dan Crozier, MC	Presentation of report of the Commission on Epidemiological Survey for the commission director; USAMRIID research program.
6 Jun 69 Medical Microbiology Course The University of Vermont Burlington, Vermont	Colonel Dan Crozier, MC	Medical Defense Against BW.
9 Jun 69 Medical Seminar Walter Reed General Hospital	Dr. William R. Beisel Major Kenneth A. Woerber, MC Major John L. Winnacker, MC	Hormonal Responses to Stress.
11 Jun 69 Global Medicine Course Walter Reed Army Institute of Research	Dr. William R. Beisel Colonel Joseph F. Metzger, MC	Early Effects of Infectious Dis- eases and Approaches to Early Diagnosis.

<u>Date and Group or Individual</u>	<u>Individual(s) Participating</u>	<u>Subject</u>
12-13 Jun 69 Medical Advisory Committee Deseret Test Center Fort Douglas, Utah	Colonel Dan Crozier, MC	Medical Problems of BW.
17 Jun 69 National Animal Disease Laboratory, U. S. Department of Agriculture Ames, Iowa	Lt Colonel John D. Marshall, Jr., MSC	Relationship between Diseases of Wild and Domestic Animals: Model 2 - <u>Pasteurellosis.</u>
25 Jun 69 Training Program in Oral Science Massachusetts Institute of Technology, Cambridge, Massachusetts	Dr. William R. Beisel	Nutrition and Infection.

APPENDIX D

PUBLICATIONS OF U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

FISCAL YEAR 1969

1. Airhart, J., G. S. Trevino, and C. P. Craig. 1969. Alterations in immune responses by attenuated Venezuelan equine encephalitis vaccine. II. Pathology and soluble antigen localization in guinea pigs. J. Immuno., In press.
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8. Elsberry, D. D., D. A. Rhoda, and W. R. Beisel. 1969. Hemodynamics of staphylococcal B enterotoxemia and other types of shock in monkeys. J. Appl. Physiol., In press.
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Director of Medical Research, US Army Chemical Research and Development Laboratories, Edgewood Arsenal, Maryland 21041	1
Medical General Laboratory (406), APO San Francisco, California 96343	1
Life Sciences Division, Army Research Office, Arlington, Virginia 22204	1
Director of CBR and Nuclear Operations, Office of the Assistant Chief of Staff for Force Development, US Army, Washington, D. C. 20310	1
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US Army CBR Weapons Orientation Course, Dugway, Utah 84022 (ATTN: Librarian)	1
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Director Biological Operations, Safety Division, Pine Bluff Arsenal, Arkansas 71603	1
US Army, Chemical Corps Information and Liaison Office, APO New York, New York 09757	1
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Department Nuclear, Biological and Chemical Services, Medical Field Service School, Fort Sam Houston, Texas 78234 (ATTN: Chief, Biological and Chemical Sciences)	1
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Suffield Experimental Station, Ralston, Alberta, Canada	1
The Surgeon General, Department of the Navy, Washington, D. C. 20390	1
Chief, Naval Operations (OP922F2), Department of the Navy, Washington, D. C. 20350	1
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USAF School of Aerospace Medicine, Brooke Air Force Base, Texas 78235	1
The Surgeon General, Department of the Air Force, Washington, D. C. 20333	1
The Surgeon General, US Public Health Service, Washington, D. C. 20201	1
US Public Health Service, Communicable Disease Center, Atlanta, Georgia 30333 (ATTN: Librarian)	1
Armed Forces Epidemiological Board	
President	1
Commission on Epidemiological Survey	23
Director, Commission on Immunization	1
Director, Commission on Influenza	1

Director, Commission on Radiation and Infection	1
Director, Commission on Rickettsial Diseases	1
Director, Commission on Viral Diseases	1
Commanding Officer, US Naval Unit, Fort Detrick, Maryland 21701	1
US Public Health Service Liaison Officer, Fort Detrick, Maryland 21701	1
Fort Detrick, Maryland 21701	
Technical Director	2
Director, Biological Sciences Laboratory	1
Chief, Medical Sciences Laboratory	1
Chief, Medical Investigation Division	1
Chief, Plans and Readiness Operations Office	1
Documents Room	2
US Army Medical Research Institute of Infectious Diseases	
Each Division	8
Library	2
Contractors	16